ISOLATION, PURIFICATION AND CHARACTERIZATION OF INULIN AND FRUCTOOLIGOSACCHARIDES FROM *CHICORIUM INTYBUS* AND INULINASE FROM *ASPERGILLUS NIGER*.

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ABSTRACT

Inulin is a non-digestible carbohydrate fructan polymer consisting mainly of β (1→2) fructosyl fructose links. Enzymatic hydrolysis of inulin by inulinase results in the production of low D.P (degree of polymerization) oligosaccharides also called fructo-oligosaccharides (FOS).

Isolation of inulin from chicory root (*Chicorium intybus*) was achieved by first, extraction using deionized water (60^0C), followed by carbonation (0.1 M $Ca(OH)_2$ and CO_2 gas). This was filtered in order to remove the non sugars, thereafter, treated successfully with polyamide 6 powder. A cation exchanger and an anion exchanger were used to further exclude other components such as tannins and pigments. The extracted inulin was quantified using the Somogyi-Nelson colourimetric assay. Chicory root (207 g, 30 % being water) yielded 30 g of the raw extract. A 100 mg of the raw extract was assayed and found to contain 11 % yield of inulin which was 80.2 % in purity and 4 % free fructose. Analysis of the crude and purified inulin extracts on the MALDI TOF spectrometry showed the samples to have a DP of 2 to 22 and 2 to 27 respectively.

Maximum inulinase production from *Aspergillus niger* grown on inulin was observed after 60 hours. The enzyme activity was found to be 1.168 U/ml with a temperature and pH optimum of 30 °C and 7.7 respectively. The enzyme proved to be unstable as it progressively lost its total activity during attempts at purification.

Key words: inulin, chicory, fructooligosaccharides, *Aspergillus niger*, inulinase.

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1-FFT= Fructan:fructan fructosyl transferase
1-SST= Sucrose:sucrose fructosyl transferase
6G-FFT= Fructan:fructan 6G-fructosyltransferase
6-SFT= Sucrose:fructan 6-fructosyltransferase
ANTS= 8-aminonaphthalene-1,3,6-trisulphonic acid
ATP= Adenosine tripphosphate
BSA= Bovine serum albumin
$Ca(OH)_2$= Calcium hydroxide
$CaCO_3$= Calcium carbonate
CO_2= Carbon dioxide
Conc= Concentration
DP = Degree of polymerization
DEAE= Diethylaminoethyl cellulose
DMSO= Dimethylsulphoxide
dH_20= Distilled water
[F]= Fructose concentration
FACE= Flourophore-assisted carbohydrate electrophoresis
FEH= Fructan exohydrolase
$FeSO_4.7H_2O$= Ferrous sulphate heptahydrate
Fn= Fructose with n ranging from 2 to 10
FOS= Fructooligosaccharides
GFn= Sucrose with n ranging from 2 to 10
GIP= Glucose dependant insulinotropic polypeptide
HFCS= High fructose corn syrups
HPLC= High performance liquid chromatography
HPAEC-PAD= High pressure anion exchange chromatography with pulsed amperometric detector.
[I]= Inulin concentration
KCl= Potassium chloride
kJ/g= Kilojoules per gram
M= Molar
MALDI TOF= Matrix assisted laser desorption ionization time of flight mass spectrometry
$MgSO_4.7H_2O$= Magnesium sulphate heptahydrate
ml/min= milliliter per minute
mRNA= Messenger RNA
m/z= Mass to charge ratio
NADH= Nicotinamide adenine dinucleotide
NF-kB= Human NF-kappa-B transcription factor p65 subunit mRNA
$NH_4H_2PO_4$= Ammonium dihydrogen phosphate
Pol= polyamide 6 powder
rpm= Revolutions per minute
SDS-PAGE= Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLS= static light scattering

TAG= Triacylglycerol
TLC= Thin layer chromatography
TNFα= Tumour necrosis factor- α
UHFGS = Ultra high fructose glucose syrups
VLDL= Very low density lipoprotein
VLDL-TAG= Very low density lipoprotein-Triacylglycerol

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Chapter 1

Literature review

1.1 Introduction

Chicory (*Cichorium intybus* L), a species of the Asteraceae family, is a perennial plant of about 1 to 1.8m in height (Figure 1.1) (Hazra *et al*., 2002). The crop is widely cultivated in a number of temperate regions around the world including South Africa, but has its origins in Europe, central Russia and western Asia (Koch *et al*., 1999). It is a vegetable known as endive (American) or witloof (Australian). Chicory is grown for a number of reasons in different countries. In Brazil for example, it is cultivated for the production of leaves, while in India, South Africa and Belgium roasted chicory is mixed with coffee seeds for the preparation of coffee powder. In recent years, however, Belgium has been active in processing the root for the production of inulin (fructose polymer) and its hydrolysis products such as oligofructose and fructose (Figueira *et al*., 2003; Zhang ***et al***., 2003; Gadgoli and Mishra, 1997; Baert and Van Bockstaele, 1992).

Figure 1.1: Chicory roots after harvest. ©J.Dames 2003

The use of chicory roots for human consumption can be traced as far back as 2000 B.C from the *Ebers Papyrus* (ancient Egyptian medical document), where Egyptians on the banks of the Nile cultivated chicory and used it to aid digestion. The popularity of chicory as a herbal plant in Europe came after its documentation by Pedanios Dioscoride, a Greek doctor in the Roman army, who was the first to mention its curative powers. It subsequently became common to employ chicory root extracts as therapeutic interventions for liver, kidney and stomach complaints (Luckman and Rossouw, 2003).

The commercialization of roasted chicory roots as coffee additives was first observed in the 18th century in Europe, the same period where the first commercial chicory processing French factories appeared. This massive production of roasted chicory was largely prompted by Napoleon's continental blockade of English shipping, which indirectly contributed towards the origins and development of the South African chicory industry (Luckman and Rossouw, 2003).

1.1.1 Development of the South African chicory industry

During the early years of the 18th century, the Napoleonic blockade impacted negatively on the lines of trade with coffee sources. Such interferences prompted the coffee industry to look elsewhere for alternatives, which were subsequently realized in chicory. Unknowingly to many, this was to be the beginning of the development of the South African chicory board (Young, 1958).

Chicory was locally grown on a commercial scale for the first time during the eighteen nineties. By the early nineteen hundreds chicory plantations had extended to areas like Paarl, Alexandria, Queenstown and East London. This growth was however, not without problems, as steady supplies in sufficient quantities (for factory processing) were not forthcoming due to increasing demands. The end of the First World War (the very stimulus of the industry) made it possible for importers (particularly Belgium and Holland) to compete with local growers (already in fierce competition). Overseas growers sold their

crop at comparatively low prices which forced many local growers to revisit a 1906 suggestion concerning co-operative marketing (Young, 1958).

It is due to these market complexities that on the 29th September 1926 the Alexandria co-operative chicory growers' society was established and registered as an unlimited company. The main objective of the society was to minimize competition among local producers and to stabilize the already volatile market (Young, 1958).

On the 1st October 1993, the chicory board was discontinued and replaced by a public company, which issued all the company's shares to registered chicory growers (Agriculture in South Africa, 1994).

1.1.2 Current state of affairs

Of all the chicory growing countries, South Africa is still one of the few countries where chicory is solely grown for coffee purposes. Northern and central European countries (particularly Belgium, the Netherlands and France) exploit the root for inulin and fructose syrups (D'Egidio *et al*, 1998; Agriculture in South Africa, 1994).

Despite the fact that most European companies produce more chicory and inulin, maximum production of these products is hampered by limited space, cost of the raw materials, land and labour availability and transportation. As the fifth largest chicory producing country (Table 1.1), South Africa has the means and capacity to compete and benefit from participating in inulin related markets as land and labour are not limited. Moreover, it has the capacity, infrastructure and expertise as the chicory industry is well established. Presently, only 6000 hectares (along the south east coast) are in use due to the current national trade of 63000 metric tons of chicory. Inulin exports by Chicory S.A could result in the acquisition of 120000 hectares, and as a result more jobs and revenue would become available, with the subsequent upliftment of the local communities (Luckman and Rossouw, 2003).

Table 1.1: Major chicory and inulin producing countries ((Luckman and Rossouw, 2003).

World chicory production			World inulin production		
Country	Tonnes	%	Country	Tonnes	%
Belgium	650000	53.8	Belgium	600000	75
Netherlands	250000	20.6	Netherlands	200000	25
France	120000	9.9			
India	90000	7.4			
South Africa	63000	5.2			
Poland	28000	2.3			
Spain	8000	0.007			
Other	4000	0.0035			

Presently chicory is predominantly grown in the coastal area of the Eastern Cape, mostly in the districts of Alexandria, Paterson, the Gamtoos area, parts of Peddie, Albany and Bathurst. After harvesting the roots are dispatched to Chicory S.A for processing (Figure. 1.2) (Luckman and Rossouw, 2003; Agriculture in South Africa, 1994).

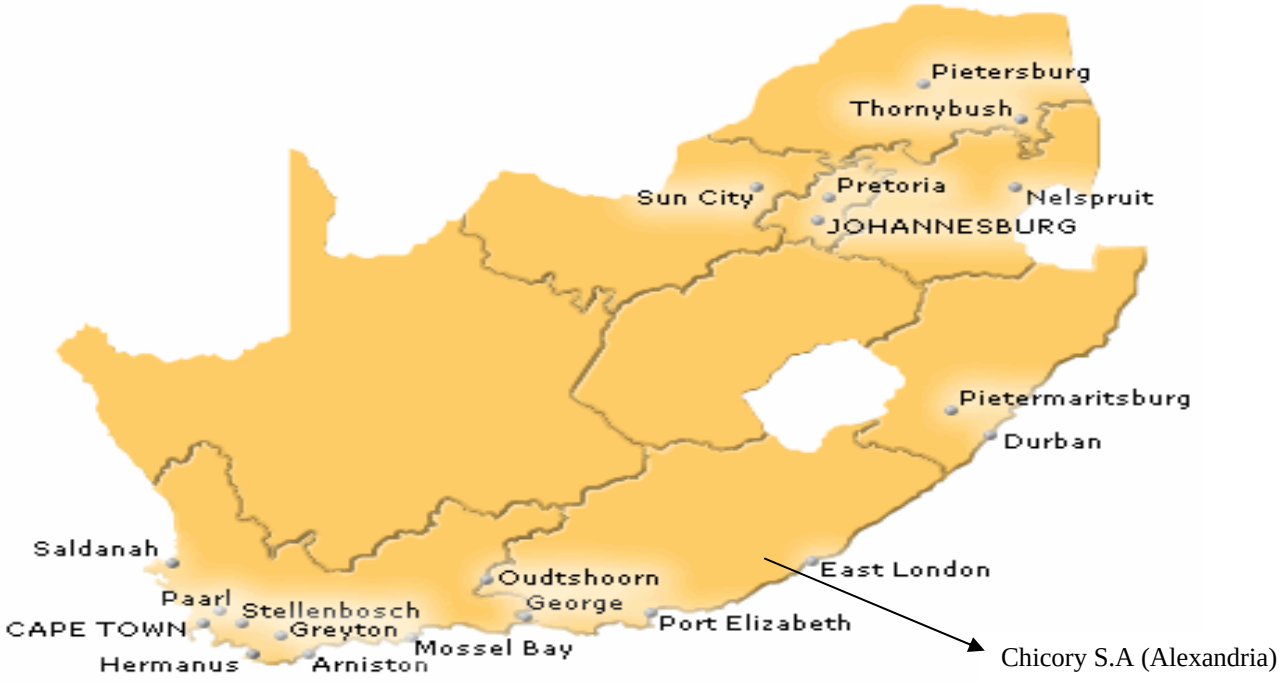

Figure 1.2: Location of Chicory S.A on the Eastern Cape province of South Africa. (Safari-SA, 2003).

At the company's central drier the roots are graded, washed, dried, diced and roasted to supply the country's biggest coffee manufacturers. Thus, Chicory S.A's main activities focus on supplying the coffee industry and its related subsidiaries with ground chicory for blending with coffee. The company recently embarked on a programme to start exporting some of their product to the U.S.A. (Agriculture in South Africa, 1994; Luckman and Rossouw, 2003).

Apart from the root's beneficial carbohydrate components, chicory's addition to coffee ensures the enhancement of flavour and aroma, while providing strength to the brew. Moreover coffee blended with chicory is comparatively cheaper than pure coffee. As a result almost 88 % of all coffee products produced locally contain chicory as an additive (Agriculture in South Africa, 1994; Luckman and Rossouw, 2003).

1.2 General uses of chicory roots

The root is versatile in its application. Apart from its use as a major component of indigenous drugs, it has been greatly utilized as a food and feed source (Zafar and Ali, 1998; Koch *et al*., 1999).

As a medicinal substituent, chicory has been proven to have anti-hepatotoxic effects. Due to its function, the liver is generally susceptible to toxins that pass through for transformation purposes. During this process, some toxins are known to generate free radicals that react with oxygen and subsequently constituting a peroxy radical that contributes to the hepatotoxicity, which is generally characterized by an increase in serum transaminase activity (Zafar and Ali, 1998).

Pharmaceutical and food industries often use antioxidant compounds as additives in their products to prevent lipid peroxidation (auto-catalytic chain reaction), as it adversely alters flavour and nutritional composition of fat rich products (Papetti *et al*., 2002). Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butylated hydroquinone (TBHQ) and propyl gallate have been widely used as a

preventative measure for the oxidation of food and beverages. The advantage of using synthetic antioxidants lies on the fact that they are highly active and cheap. Moreover, they offer convenience in that they are odourless, colourless, and tasteless. One of the few commercial difficulties they present is that they are only active at low temperatures. They are not only ineffective at high temperatures (150-200°C), but they decompose and evaporate with steam, especially when a large quantity of water is expelled from foods during deep frying and baking (Zhang *et al*., 2004). Furthermore, they have been found to contribute in promoting carcinogenesis, and established to be generally unsafe for human health (Papetti *et al*., 2002). As an alternative, plant material is known to exhibit anti-oxidant activities, in particular a variety of chicory known as Treviso red chicory (*Chicorium intybus* var. silvestre) is able to counteract lipid peroxidation (Papetti *et al*., 2002).

The majority of the antioxidant activities found in *Chicorium intybus* var. silvestre may be contributed by compounds such as flavonoids, isoflavone, flavones, anthocyanin and catechins rather than by other systems such as vitamin C, E and β-carotene, as is the case with most plants. Besides, *Chicorium intybus* var. Silvestre is not known to contain the abovementioned vitamins (Osman *et al*., 2004).

The leaves and heads of the root are mostly appreciated for their slight bitter taste (due to sesquiterpene lactones) and normally used as a salad crop and vegetable. This is particularly common in some European countries (e.g. Belgium) (Zhang *et al*., 2003; Poli *et al*., 2002). Prior to grinding the root, it is dried, roasted and used as a coffee substitute or extender, a feature employed by the coffee industry (Gadgoli and Mishra, 1997).

However, apart from all the attributes chicory has to offer, inulin remains one of the most biotechnologically important components of the root, due to commercial benefit (Ritsema and Smeekens, 2003).

1.3 Fructans as alternative storage polymers

Plants require energy for survival and proper maintenance of cellular processes. They derive their energy through photosynthesis, a process by which light energy is used to convert CO_2 to highly reduced carbon compounds such as carbohydrates. The resultant carbohydrates are then stored in the form of storage polysaccharides for later use, should the need arise. Most plant cells contain starch (a polysaccharide mixture of amylose and amylopectin) as a major reserve carbohydrate. The exploitation of starch for energy release is achieved through a cascade of reactions that involve the conversion of glucosyl residues to glucose 6-phosphate, which can enter the glycolytic pathway thereby producing ATP through substrate level phosphorylation. Substantial ATP is generated through oxidative phosphorylation under aerobic conditions. In times where carbohydrates are taken up or manufactured within the cell, glucose 6-phosphate can be converted to starch and the carbohydrate stored (Smith and Wood, 1991).

Although starch is the most common reserve polysaccharide in the plant kingdom, exceptions exist in some species where a different polysaccharide is used for such purposes (Smith and Wood, 1991). Fructans are among the most widespread alternative storage materials found in a range of temperate, mainly perennial monocots within the Liliflorae and Glumiflorae together with dicots of the Asterales (e.g *Chicorium intybus*), Campanales and Boraginaceae (Meier and Reid, 1982 Lewis, 1984).

The most striking feature of fructans is that they do not only confer storage properties, but play a role in assisting plants to adjust to ever changing climatic conditions (De Roover *et al*., 2000).

1.3.1 Sucrose usage and ultimate fructan accumulation

A major part of the organic carbon produced by plants during photosynthesis is channelled into the synthesis of sugars, which are the major carbon compounds exported from photosynthetic sources to storage organs. In most plants, the transported sugar is sucrose (a

non-reducing disaccharide) in which glucose and fructose are α1→ β2 linked (Sturm, 1999; Druart *et al*., 2001).

The driving force behind sucrose transport is through a succession of multifaceted reactions which include, amongst others, turgor pressure gradient promoted by a sucrose concentration gradient, and by the energy dependent transport of sucrose through membranes (Ho and Baker, 1982; Patrick, 1990). Depending on the physiological activities and the biological demands of the non-photosynthetic tissues, different biochemical pathways in various subcellular compartments are fed with sucrose which may be channelled into the glycolytic and tricarboxylic acid pathways for the generation of ATP and NADH (Sturm, 1999). The carbon of the disaccharide may be directed towards the biosynthesis of primary metabolites destined for tissue growth and development. For long-term storage purposes, sucrose may become converted into polymeric compounds such as starch, triacylglycerides, polypeptides or secondary metabolites that provide plants with the means to cope with pests and predators (Sturm, 1999). The resultant accumulation of sucrose in storage organs ultimately leads to fructan synthesis and storage, either for long or short term purposes (De Roover *et al*., 2000).

1.3.1.1 Role of fructans in plants

The accumulation of fructans may be attributed to adverse climatic conditions (such as hypoxia, low temperature, drought and high CO_2 concentrations). For example, during hypoxia, **a** condition characterized by the absence of oxygen supply, the metabolic activity of roots (as storage organs) for carbohydrate storage is reduced as a result of retarded carbohydrate oxidation by the lack of electron acceptors [NAD (P^+), oxygen], while the photosynthetic organs are largely unaffected. Sucrose export from the photosynthetic regions into the roots remains unchanged, thus accumulating large amounts of fructose residues (Albrecht *et al*., 1993).

An increase in glucose, fructose and sucrose is observed in the roots of plants found in areas with sporadic rainfall. During drought stress, a shift from photosynthesis to

carbohydrate synthesis is favoured by the activation of sucrose phosphate synthase through reversible protein phosphorylation. The induction of sucrose synthesis during this time is generally as a response to maintain osmoregulation (facilitated by water soluble carbohydrates) under control (De Roover *et al*., 2000). Plants that have optimum osmoregulation are capable of maintaining membrane integrity and thereby preventing protein denaturation. As a result of all the extreme climatic conditions, the sucrose:sucrose fructosyltransferase (1-SST) gene is induced and 1-SST activities increase, thus resulting in an increased accumulation of fructans, which make the plant tolerant to unfavourable weather changes. As a result, subcellular structures are maintained in aqueous environments (De Roover *et al*., 2000).

1.3.2 Types of fructans

Fructans are polysaccharides comprised of repeating fructose units and consist of a glucose molecule normally attached at the beginning of the polymer. Different fructans are found in plants either separately or mixed and they have two different glycosidic linkages, namely β (2→1) linkages (a common feature in inulin type), β (2→6) (normally found in levans) and graminan, comprised of both β (2→1) and β (2→6) (Vereyken *et al*., 2001; De Roover *et al*., 2000).

Inulin is a linear polyfructan that is mainly comprised of β (2→1) linked fructose units with a degree of polymerization (DP) ranging between 2 and 60 (Figure 1.3) (Druart *et a*l., 2001). It is found from a variety of sources such as Jerusalem artichoke, chicory (high inulin content), onion, garlic, banana, asparagus and leek. Examples in this group include the trisaccharide 1-kestose (isokestose), which is the shortest inulin molecule (Figure. 1.4) (Vijn and Smeekens, 1999). Bifurcose, a tetrasaccharide, is an example of a mixed levan and comprised of, β (2→1) and β (2→6) linkages (Figure. 1.5) (Sprenger *et al*., 1997). Levan is a homopolymer that is distinctive of β (2→6) linkages and is predominantly found in some grasses particularly *Dactylis glomerata* L (Figure. 1.6) (Park *et al*., 2003; Bonnett *et al*., 1997).

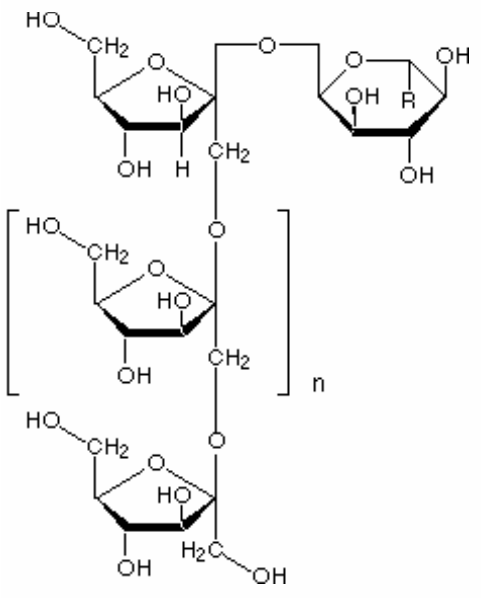

Figure 1.3: Simplified structure of inulin, fructose moieties are linked in a repeating β 2 → 1conformation. R = CH_2OH and n = approximately 50. Vandamme and Derycke (1983).

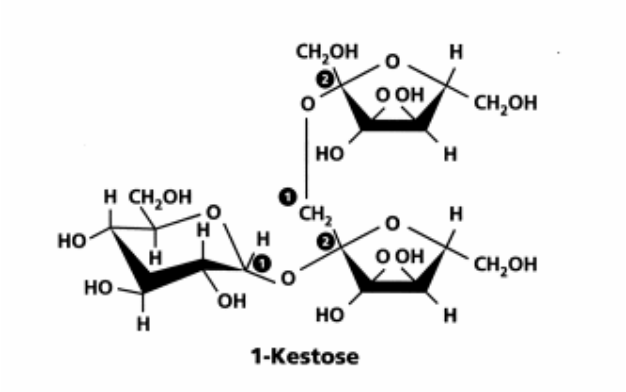

Figure 1.4: The trisaccharide 1-kestose is the shortest inulin molecule consisting of β (2⟶1) linkages between fructose residues and is normally terminated by a glucose molecule (Vijn and Smeekens, 1999).

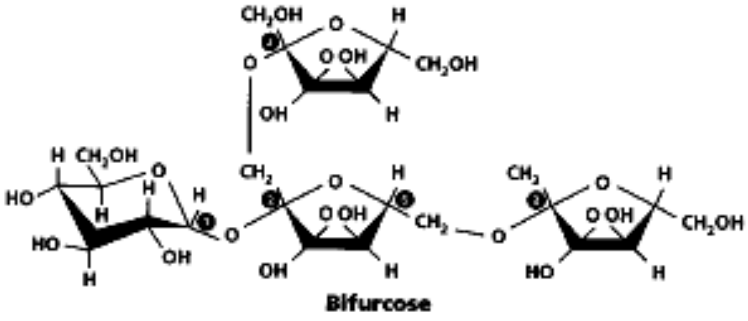

Figure 1.5: A mixed levan bifurcose consists of a (2⟶1) and a (2⟶6)-linked β-D-fructosyl unit linked to sucrose (Vijn and Smeekens, 1999).

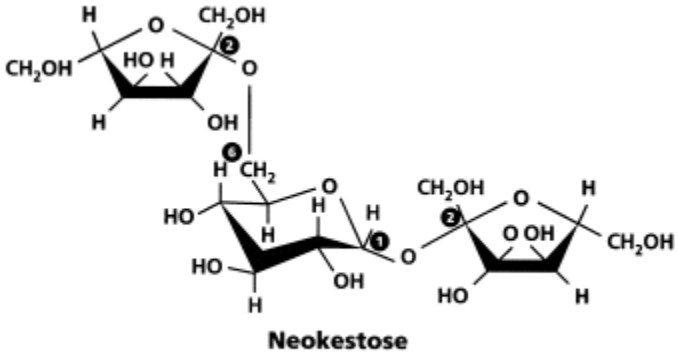

Figure 1.6: A branched levan neokestose consists of β (2→6) glycosidic linkages (Vijn and Smeekens, 1999)

1.3.2.1 Fructan biosynthesis

Two enzymes working in concert are involved in the formation of fructans, sucrose: sucrose fructosyltransferase (1-SST) and fructan: fructan fructosyl transferase (1-FFT) (Stahl *et al.*, 1997). 1-SST initiates the reaction by forming the shortest inulin (1-kestose, a trisaccharide) from sucrose (Ritsema and Smeekens, 2003). 1-FFT is mainly responsible for chain elongation, this is effected through the catalysis of fructosyl transfer from one fructan molecule to another. During this stage 1-kestose (the product of 1-SST), or at times fructans of higher DP are used as fructose donors destined for addition towards sucrose or an assortment of other fructans (Stahl *et al*., 1997; Ritsema and Smeekens, 2003).

The synthesis of more complex fructans (e.g. mixed or branched fructans), requires the use of additional enzymes such as fructan:fructan 6G-fructosyltransferase (6G-FFT) and sucrose: fructan 6-fructosyltransferase (6-SFT) which still act in conjunction with 1-SST (Ritsema and Smeekens, 2003). During the production of neo-series inulin (a trisaccharide where a glucose moiety is inserted between two fructosyl residues), the product of 1-SST-initiated reaction, 1-kestose, is used by 6G-FFT as a substrate, where a fructosyl residue is transferred to a glucose moiety of sucrose through a β (2→6) glycosidic linkage forming neo-kestose. The synthesis of branched fructans (e.g. bifurcose) requires 6-SFT and uses 1-kestose to introduce a branch thus forming bifurcose (Vijn and Smeekens, 1999).

1.3.2.2 Fructooligosaccharide (FOS) accumulation

Reactions preceding high DP fructan synthesis and conglomeration normally result in the formation of fructooligosaccharides (a combination of short and intermediate inulin chains), and three sugars collectively form this group, namely: 1-kestose (β-D-fru-$(2\rightarrow1)_2$-α-D-glucopyranoside, GF2), nystose (β-D-fruc-$(2\rightarrow1)_3$-α-D-glucopyranoside, GF3) and fructofuranosylnystose (β-D-fru-$(2\rightarrow1)_4$-α-D-glucopyranoside, GF4) (L'homme *et al*., 2003). FOS formation is as a result of a successive set of discontinuous reactions (e.g. $GF_n + GF_n \rightarrow GF_{n-1} + GF_{n+1,}$ $n < 4$, and in part from $GF_n + GF \rightarrow GF_{n+1} + G$) that generally lead to inulin constitution (particularly in chicory). The DP is probably determined by substrate availability (Kim *et al*., 1998).

1.4 Physico-chemical properties of chicory inulin

Commercial chicory inulin is available in three different compositions that are nonetheless found in native inulin (that which is extracted from fresh roots and has not been fractionated to remove sugars with a low DP). In its pure form inulin is a white, odourless powder that has no taste at all, depending on the fractionated type (Franck, 2002 b). Standard inulin has fructosyl linkages that range from 2 to 60, with 10 % sweetness due to fructooligosaccharides that have not been removed, and show inadequate solubility. Whereas, the fructose residues in high performance inulin vary between 10 and 60 with an average DP of 25, fructooligosaccharides have been completely removed and therefore the product is tasteless with poor solubility in water at room temperature. Oligofructose powders on the other hand are moderately sweet (35% as sweet as sucrose) and soluble in water. These are very short inulin chains having a polymerization degree that is less than 9. They have a chemical structure that can be represented as $GF_n + F_n$, which infers that they are found in the presence of free fructose (F_n) (hence the sweetness) (Franck, 2002 b; Bekers *et al*., 2002).

1.4.1 Utility in foods

The food industry has found major applications for the manufacture of inulin derived or associated products, mostly because it combines easily with other food ingredients and contributes towards improved organoleptic (impression produced by any substance on the organs of touch, taste or smell) qualities without altering any flavours (Franck, 2002 b). Production of bread requires the use of fats as they delay the staling process, whilst giving it volume and a softer texture. Dietary fat intake however, has been linked to increased blood cholesterol with subsequent onset of heart diseases (O'Brien *et al*., 2003; Harris *et al*., 2003). As an alternative, O'Brien *et al* (2003) demonstrated that high performance inulin has excellent fat mimicking characteristics when combined with water (as it forms a gel), and that bread incorporating inulin as a fat substitute had similar characteristics in quality when compared with bread containing fat.

As sweetening agents, fructooligosaccharides are not known to cause tooth decay and moreover, do not have the bitter aftertaste that is characteristic of some artificial sweeteners (e.g. saccharin). Aspartame on the other hand is claimed to be two hundred fold sweeter than sucrose with a low caloric value, and is regarded as a non carbohydrate sweetener (Passador-Gurgel *et al*., 1996; Vandamme and Derycke, 1983). Furthermore, problems associated with corpulence and artherosclerosis as a consequence of sucrose usage are not observed with oligosaccharides. As a complication for diabetic patients, sucrose has its draw backs in insulin deficient systems, and as a result fructooligosaccharides are emerging as alternatives, as they are metabolized in low levels without the requirement for insulin (Passador-Gurgel *et al*., 1996). An overview of the functionality of inulin and fructooligosaccharides in food applications is presented in table 1.2.

Recently, it has been shown that modified inulin can be exploited as a chelating agent, a detergent co-builder and pharmaceutically as a carrier for drug delivery (i.e. for drugs destined for the large intestine). Drug delivery to the large intestines does not always reach the targeted sites due to the intestinal hydrolytic enzymes which interfere with the coating and result in such drugs emptying before reaching the colon. As inulin is not susceptible to

hydrolytic enzymes, most pharmaceutical companies have been developing colon drugs encapsulated with nondigestible polysaccharides known as prodrugs (e.g. Pulsincap^{TM}) (Kim and Wang, 2001; Vandamme *et al*., 2002).

These drugs are thus able to reach the large intestine where fermentation of the capsule takes place and the drug released (Kim ***et al***., 2001). Figure 1.7 is a schematic representation of the Pulsincap system.

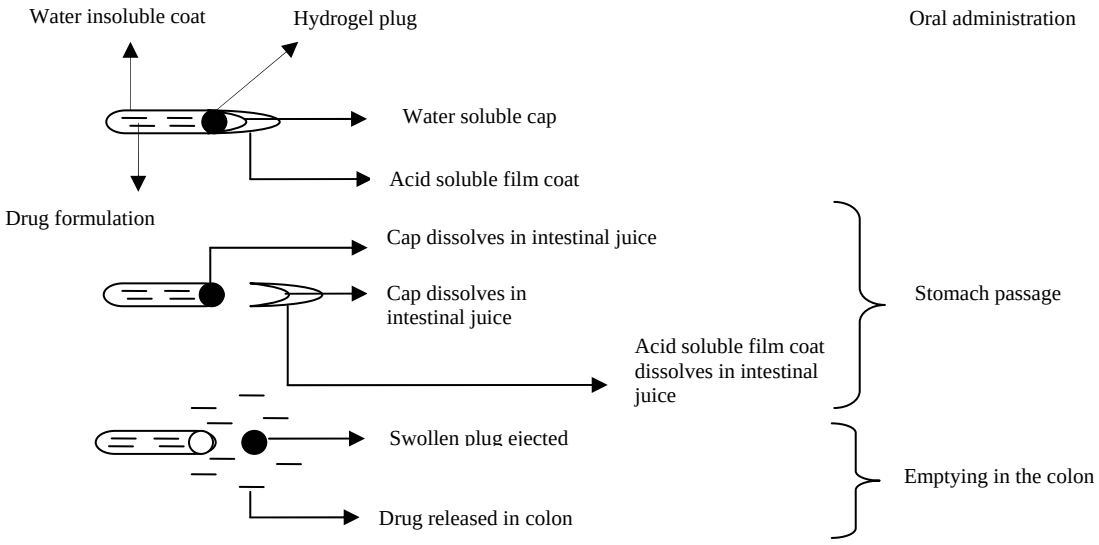

Figure 1.7: Schematic representation of the Pulsincap system. Adapted from Vandamme *et al*., (2002).

Table 1.2: An overview of the functionality of inulin and FOS in food applications Franck, 2002 b

Application	Functionality	Dosage level inulin (% w/w)	Dosage level oligo (%w/v)
Salad dressings	Fat replacement Body and mouthfeel	2-10	-
Meat products	Fat replacement Texture and stability Fibre	2-10	-
Dietetic products and meat replacers	Sugar and fat replacement Synergy with sweeteners Low caloric value Body and mouthfeel Fibre and prebiotic	2-15	2-20
Chocolate	Sugar replacement Fibre Heat resistance	5-30	-
Tablets	Sugar replacement Fibre and prebiotic	5-100	2-10

Application	Functionality	Dosage level inulin (% w/w)	Dosage level oligo (%w/v)
Dairy products	Sugar and fat replacement Synergy with sweeteners Body and mouthfeel Foam mobility Fibre and prebiotic	2-10	2-10
Frozen desserts	Sugar and fat replacement Texture and melting Synergy with sweeteners Fibre and prebiotic	2-10	5-12
Table spreads	Fat replacements Texture and spreadability Emulsion stability Fibre and prebiotic	2-10	-
Baked goods and breads	Fibre and prebiotic Moisture retention Sugar replacement	2-15	2-25
Breakfast cereals	Fibre and prebiotic Crispness and expansion	2-25	2-15
Fillings	Sugar and fat replacement Synergy with sweeteners Body and mouthfeel Fibre and prebiotic	2-30	2-50
Fruit preparations	Sugar replacement Synergy with sweeteners Body and mouthfeel Fibre and prebiotic	2-10	2-50

1.4.2 Enzymes

On a comparative basis, fructose is up to twice as sweet as sucrose (normal table sugar), does not impact negatively on health and occurs abundantly in nature. It is for this reason that during the 1980's various industrial processes were employed to isolate fructose for application throughout the food industry. The standard procedure for high fructose syrup production involved the hydrolysis of starch into glucose and its ultimate conversion by D(+)-xylose isomerase (glucose isomerase EC 5.3.1.5) into a mixture containing 50% glucose, 42% fructose, and 8% of other sugars (Vandamme and Derycke, 1983; Passador-Gurgel *et al*., 1996).

The latest approach in the formation of concentrated fructose is the use of enzymes. This is due to the fact that enzymes are specific and the generation of undesirable by-products, as obtained with chemical conversion, is avoided. The reactions proceed under mild conditions (e.g. pH, temperature and pressure) and they are easier to immobilize on solid supports for continuous industrial hydrolysis of polymers (MacCabe *et al*., 2002).

The convenience and efficiency at which inulin can be processed by immobilized inulinases was demonstrated by Nakamura *et al*., (1995), where they showed that dahlia inulin 5% (w/v) dahlia inulin solution at pH 5, can be completely hydrolyzed to 97 % D fructose and 3 % D-glucose, over a period of 45 days utilizing a packed bed column reactor containing 8 ml of the immobilized inulinase (from *Aspergillus niger* Mutant 817) at a flow rate of 1 ml/min.

Inulinases are β-fructosidases (mainly 2,1-β-D-fructan-fructanohydrolases) that liberate fructose moieties from the non-reducing end of the polyfructan (Vandamme and Derycke, 1983). Plants and microorganisms (mostly fungi, yeasts, and bacteria) are the best known sources for inulinase production. However, differences in enzyme activities do exist and are source dependent. Plant inulinases, for example are of a single type (exohydrolases) and show slight or no invertase activities (hydrolyze sucrose into glucose and fructose) (Vandamme and Derycke, 1983; Claessens *et al*., 1990; Henson, 1989).

Even though a number of microorganisms synthesize only exo acting enzymes (as in plants), the fact that enzyme production can be induced and optimized provides numerous biotechnological possibilities. Recent developments have shifted towards the use of mutant microbes for the synthesis of both the exo- and endoinulinases (randomly cleave inulin's internal linkages to produce inulo-triose, -tetraose and pentaose as major products). These enzymes act synergistically for high yield production of fructose syrups (Nakamura *et al*., 1994; Jing *et al*., 2002). The characteristic feature of mutant microbes is their potential to synthesize enzymes with high inulinase activities. However, their invertase activities are compromised when compared to wild types, but the overall inulin/sucrose ratios (I/S) remain high (Nakamura *et al*., 1994; Jing *et al*., 2002).

Aspergillus species, particularly *Aspergillus niger* and its mutants (e.g. *Aspergillus niger* 12 and 817, as used by Nakamura *et al*., 1994) has been extensively utilized for the production of inulinases and subsequent hydrolysis of inulin. The fungus offers ease and convenience in that it secretes its secondary metabolites (product excreted by a microorganism near the end of its growth phase or during the stationary phase) extracellularly, as opposed to most bacteria which normally have their enzymes membrane bound, (MacCabe *et al*., 2002).

1.4.3 Inulin and fructooligosaccharide energy value

As beneficially healthy as these functional food ingredients (food ingredients that promote the state of health, by targeting certain bodily functions and preventing the risk of disease) are, their caloric content is very low as compared to that obtained from normal table sugar (17 kJ/g), a feature which makes inulin and oligofructose even more appealing. The reason for this observed decrease in caloric content is due to the fact that their energy value depends on their fermentability and generation of gases. Their overall energy value ultimately translates to 12 kJ/g, as measured by whole body indirect calorimetry (Roberfroid, 2002; Figueira *et al*., 2003; Cherbut, 2002).

1.5 Regulation of intestinal microflora

Due to the fact that intestinal hydrolytic enzymes only target α linked glycosides, it is easier for inulin related products (β (2→1) configuration) to pass through the small intestine to the large colon without any interference. Figure 1.8 schematically reflects inulin's stability and resistance towards different enzyme systems along the alimentary canal, starting with the salivary amylase in the oral cavity, which has no affinity towards fructans, apart from the hydrolysis of α-1→4 and β 1→4 glycosidic linkages. Intestinal enzymes don't have any pronounced hydrolytic effects, which renders the oligomers unavailable, as they can not be absorbed. The polymer is thus left available for the colonic bacteria to utilize as fuel (Roberfroid, 2002; Kandra, 2003).

Apart from the technological properties inulin and oligofructose have to offer, the most impressive feature is their effect as prebiotics which are foods that beneficially affect the gut by selectively stimulating the growth and propagation of bifidobacteria and lactobacilli with a concomitant decrease in pathogenic bacteria. This ultimately leads to a well conserved integrity of the gut microbiota (Cummings and Macfarlane, 2002; Kolida *et al*., 2002). To further support and establish inulin and oligofructose as prebiotics, Rao (1999) observed that all strains of bifidobacteria particularly *Bifidobacterium infantis*, *B. pseudo-longum*, *B. angulatum* and *B. breve*, had consistent and better growth on chicory inulin than on glucose. Accumulation of bifidobacteria in the large intestine is beneficial to human health in as far as protection against diseases and efficient gut function is concerned.

Subsequent fermentation of inulin leads to bifidobacteria secreting acids which protonate potentially toxic ammonia to produce NH_4^+ which results in the lowering of blood ammonia levels. Furthermore, metabolites that are inhibitory to a range of Gram-positive and Gram-negative pathogenic bacteria are excreted. An increase in the number of bifidobacteria results in the secretion of immunomodulators that promote immunological attack against malignant cells and vitamins of the B group (e.g. vitamin B_{12}). Vitamin B_{12} is a cofactor of methylmalonyl-CoA mutase which transforms methylmalonyl-CoA into

succinyl-CoA which enters the Kreb cycle (Gibson and Roberfroid, 1995; Kadim *et al.*, 2004). Beneficial impacts of inulin are depicted in figure 1.9.

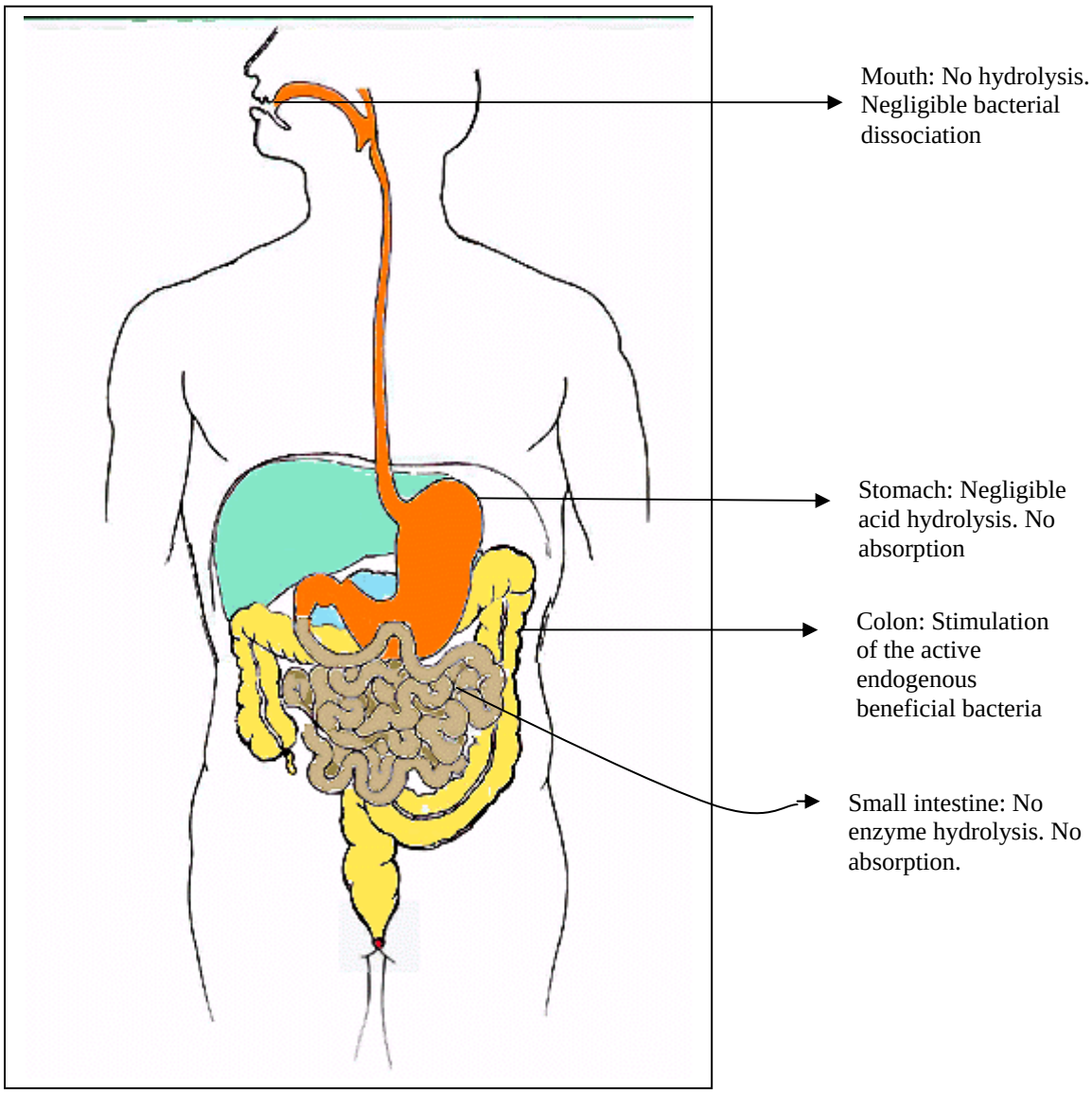

Figure 1.8: Depiction of inulin stability from hydrolytic enzymes. Gibson and Roberfroid (1995).

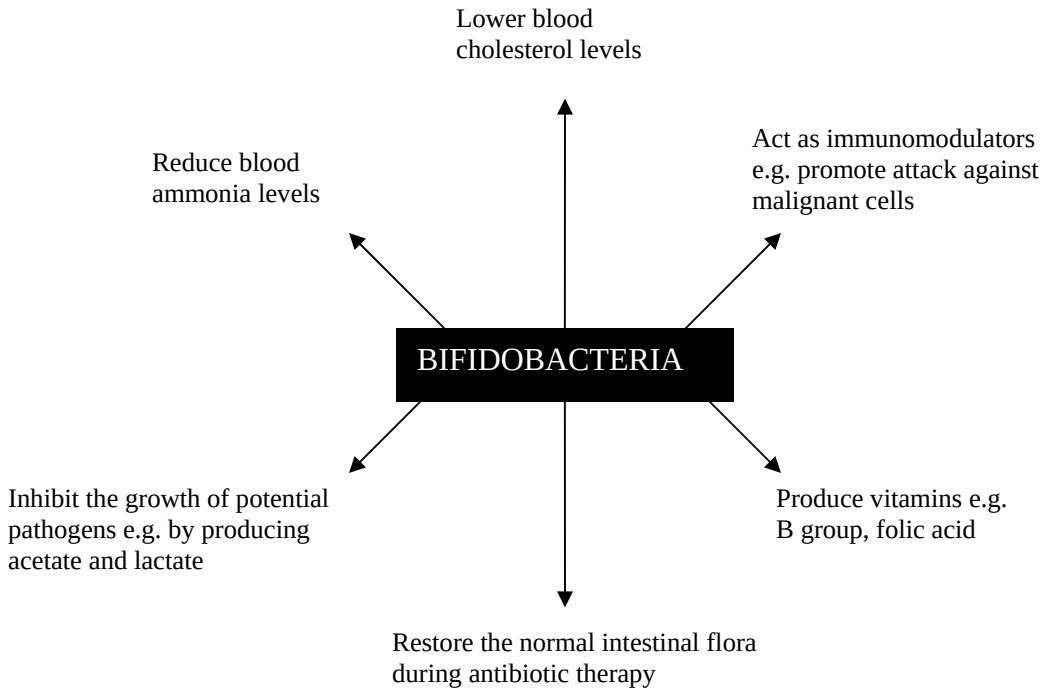

Figure 1.9: Beneficial impacts from bifidobacteria accumulation in the colon. (Gibson and Roberfroid, 1995).

1.5.1 Systemic effects of inulin on lipid metabolism

Apart from a healthy colon, peripheral physiological impacts are observed, particularly improved calcium absorption and decreased cholesterol absorption (Cashman, 2002; Kim; 2000).

Most eukaryotic cells utilize cholesterol for processes such as membrane biogenesis and cell growth. It is used as a precursor for all steroid hormones and bile acids which assist in fat digestion (Liscum and Munn, 1999; Mathews and van Holde, 1990 **a**). Cholesterol levels are in turn closely regulated by complex intracellular reactions, so as to minimize elevated blood cholesterol levels, as they are associated with atherosclerosis and other heart diseases (Liscum and Munn, 1999).

A diet rich in fat and saturated fatty acids is known to cause cholesterol related diseases. As fat replacers, non digestible carbohydrates on the other hand have been observed to result in a decrease of serum triacylglycerols and phospholipid concentrations (Harris *et al*., 2003; Delzenne *et al*., 2002). The observation in hypotriglyceridemia is mainly due to diminished plasma very low density lipoproteins concentrations (VLDL), which deliver endogenously synthesised triacylglycerols from liver to adipose and other tissues. These occur as a result of a decrease in the hepatic synthesis of triacylglycerols (TAG) as opposed to the catabolism of triacylglycerol rich lipoproteins. (Delzenne *et al*., 2002). A co-ordinated reduction in activity of all the central enzymes responsible for *de novo* lipogenesis in the liver is the most significant event in the retardation of hepatic synthesis of VLDL-TAG secretion (Delzenne *et al*., 2002).

Even though a lot of carbohydrates are essential for a healthy well being, there are instances where elevated plasma TAG levels are induced by certain sugars taken in conjunction with a fatty diet, these levels are normally observed during the fasting state. Thus following a meal, the clearance of TAG-rich lipoproteins, VLDL and chylomicrons (active in the transport of dietary triacylglycerols and cholesterol from the intestine to adipose tissue and the liver) lead to elevated fasting TAG, which subsequently leads to high cholesterol complications (Parks, 2002; Mathews and van Holde, 1990 a). Figure 1.10, summarizes events that ultimately lead to triacylglycerol accumulation.

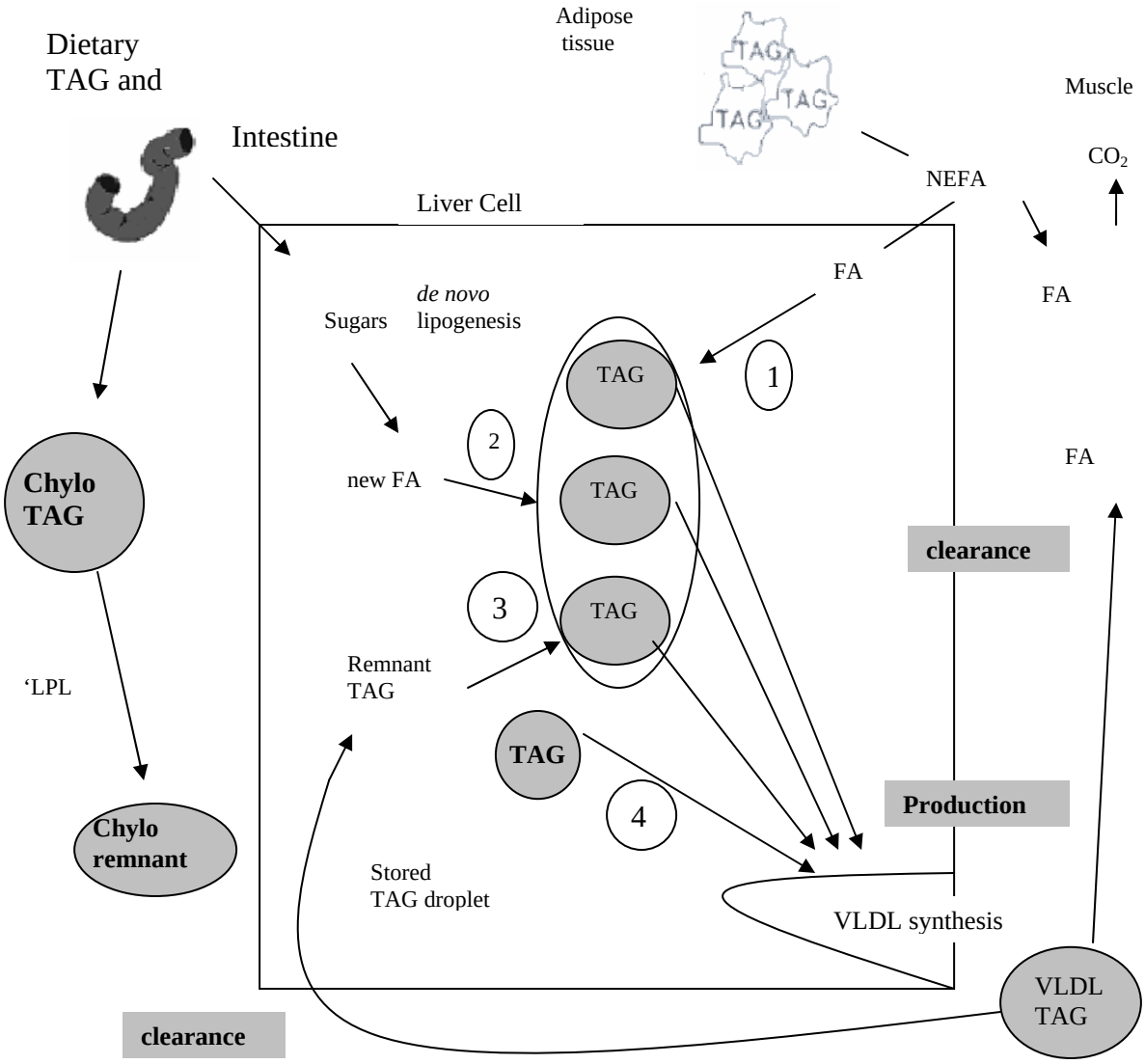

Figure 1.10: A summary of sources of fatty acids used for VLDL-TAG synthesis in the liver. VLDL, very low density lipoprotein; FA, fatty acid; TAG, triacylglycerol; LPL, lipoprotein lipase; NEFA, plasma non-esterified fatty acids (derived from adipose tissue); chylo, intestinally derived chylomicron lipoprotein particles. 1= Fatty acid derived from the plasma non esterified fatty acid pool. 2= Fatty acids derived from the *de novo* lipogenesis pathway in the liver. 3= Fatty acids originally derived from the diet that enter the liver via chylomicron remnant clearance. 4= Fatty acids that are stored in the liver in TAG droplets**.** Adapted from Parks (2002).

1.5.1.1 Cholesterol management using inulin

Despite the fact that some products of inulin fermentation tend to influence TAG and cholesterol synthesis, it is still unclear why acetate (one of the fermentation products) potentially down regulates fatty acid synthesis through acetyl CoA pool modulation (Parks, 2002).

Due to their indigestibility, inulin and oligofructose have the ability to influence the absorption of other carbohydrates such as glucose. Soluble carbohydrates have a direct influence on the release of glucose dependant insulinotropic polypeptide (GIP) and glucan-like peptide-1 amide (GLP-1) from the intestinal mucosa endocrine cells. In turn, these peptides drive lipid metabolism in the adipose tissue. Stimulation and inhibition of lipolysis (through lipoprotein lipase) seem to be dependant on the GIP concentration (Delzenne *et al*., 2002; Kok *et al*., 1998).

Nutritional interventions, utilizing oligofructose and inulin have showed a significant reduction in VLDL-TAG concentrations (Kok *et al*., 1998). These effects are supported by studies noting activities of lipogenic enzymes (particularly acetyl-CoA carboxylase, fatty acid synthase, malic enzyme, ATP citrate lyase and glucose-6-phosphate dehydrogenase) decreasing with as much as 50 % after inulin intake. This (oligofructose) impact may be due to a modification of lipogenic enzyme gene expression, which ultimately leads to hypotriglyceridemia. As a result, the food industry has begun using non- digestible carbohydrates as a means of enhancing the nutritional value of foods (Delzenne *et al*., 2002; Parks, 2002). Figure 1.11 shows putative mechanisms involved in carbohydrate facilitated lipid metabolism.

1.5.1.2 Proliferation of bifidobacteria and their effects in the colon

As non digestible carbohydrates, inulin and its derivatives are completely fermented in the colon thus generating lactic acid (metabolized into short chain fatty acids) and gases such as hydrogen, methane and carbon dioxide, which are utilized by bacteria (Cherbut, 2002).

Production of short chain fatty acids (mainly acetate, essential in the maintenance of a healthy colonic mucosa; propionate, connected with beneficial effects on carbohydrate and lipid metabolism; and butyrate, essential in maintaining a healthy colonic mucosa) plays a major role in influencing colonic motility (Causey *et al*., 2000; Cherbut, 2002; Nyman, 2002).

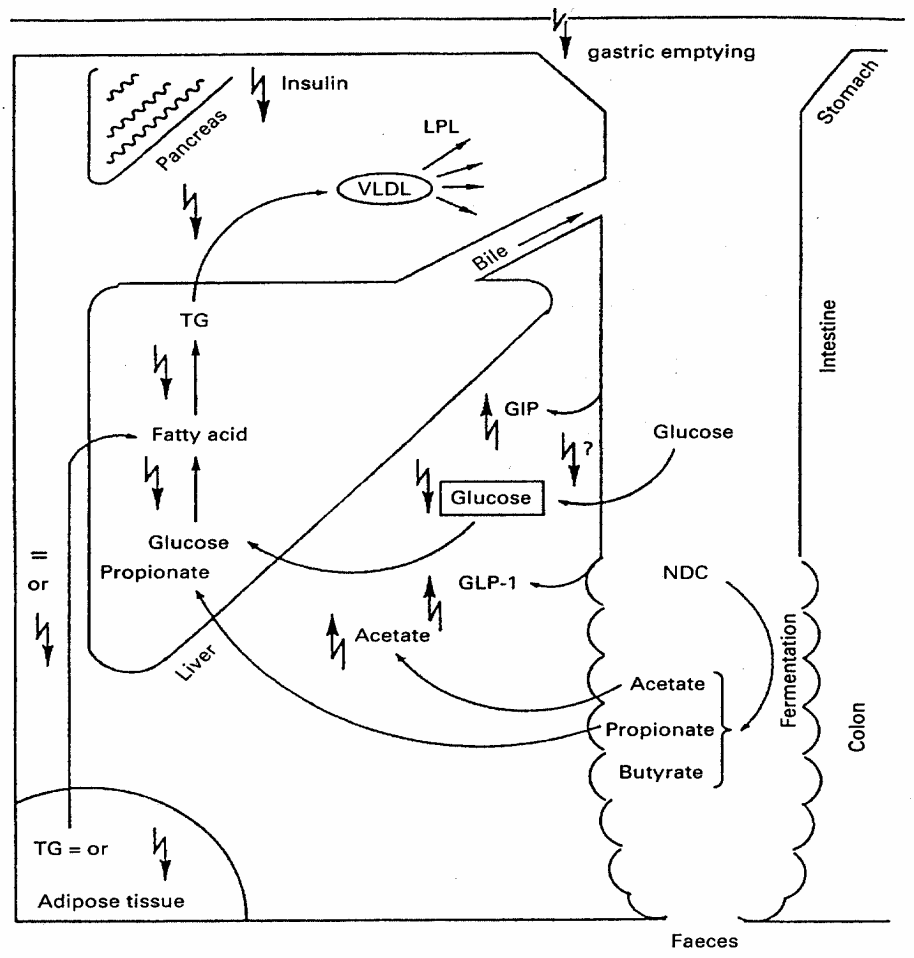

Figure 1.11: Putative mechanisms involved in lipid metabolism due to dietary inulin and oligofructose intake, including effects on gastric emptying and glucose absorption, generation of short chain fatty acids **from non** dietary carbohydrates (NDC), secretion of incretins, and subsequent actions on liver, adipose tissue and pancreatic insulin output. (Delzenne *et al*., 2002).

Interestingly, bifidobacterial propagation tends to occur at the expense of pathogenic bacteria such as *Escherichia coli*, *Clostridium perfringens*, bacteroides and other opportunistic microbes, as shown in figures 1.12 and 1.13 (Kolida *et al*., 2002; Gibson and

Roberfroid, 1995). The inhibitory effect on other bacteria mainly comes from bifidobacterial secondary metabolites such as acetate and lactate. These organic acids lower the intestinal pH thus constituting an inhospitable environment to pathogens. Furthermore, there is a possibility that certain bacteriocin-type substances that act particularly against clostridia and *E. coli* are secreted in the process (Gibson and Roberfroid, 1995). The inhibitory effect may be due to the bifidobacterial ability to secrete fructan-β-fructosidases, which are probably lacking in other intestinal microorganisms (Gibson and Roberfroid, 1995; Marx *et al*., 2000).

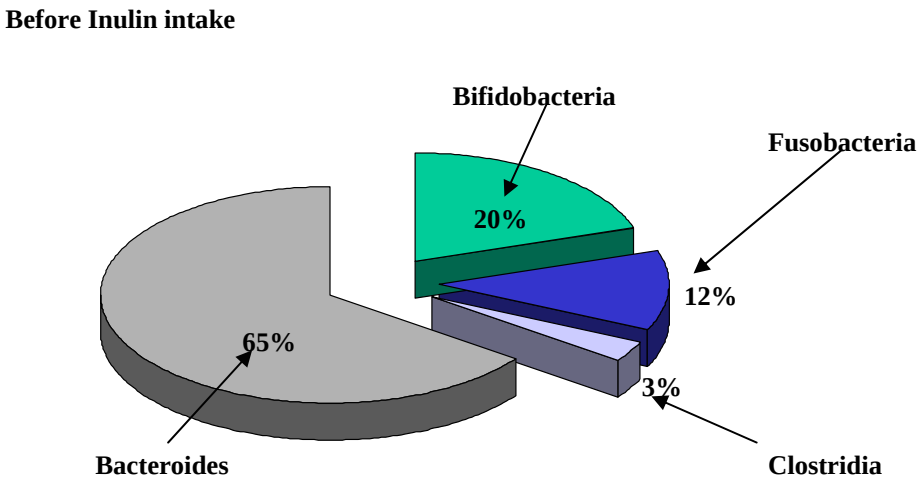

Figure 1.12: Prevalence of pathogenic bacteria before inulin intake. Adapted from Gibson and Roberfroid (1995).

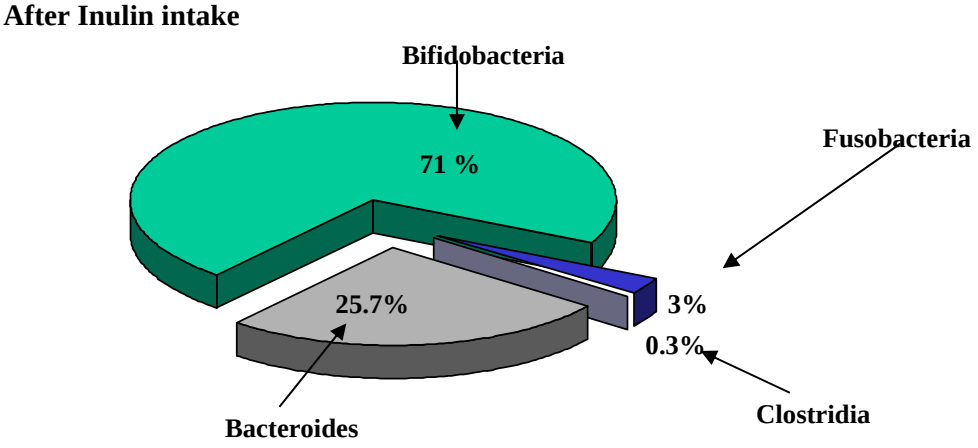

Figure 1.13: Prebiotic effect of inulin(proliferation of bifidobacteria). Adapted from Gibson and Roberfroid (1995).

1.5.1.3 Regulation of the immune system of the colon

There is strong evidence supporting the fact that gut microflora may be involved in modulating immune cells through products derived from the fermentation of non digestible carbohydrates (i.e. conversion of inulin to acetate, butyrate and propionate). Rat models have shown that improved natural cytotoxic T-cell activity results after supplementing total parenteral nutrition with short chain fatty acids (Schley and Field, 2002).

Butyrate maintains and regulates the large intestinal mucosa as a result of induction of cell differentiation, it does this by the inhibition of transcriptional factor controlling the expression of genes involved in cytokine production, cellular adhesion, inflammation and apoptosis (Ogawa *et al*., 2003; Aldieri *et al*., 2003). Furthermore, butyrate reduces the requirement of epithelial cells for glutamine, thus retaining the requirement the amino acid for other cells within the immune system (Schley and Field, 2002).

1.6 Inulin extraction

Viable processes for inulin extraction have been previously reported by Hébette *et al*., (1998) and Franck, (2002 a). Hébette *et al*., (1998), indicated a successful application of hot water (95°C) on sliced chicory roots for the isolation of inulin. The resultant crude inulin extract was then concentrated under reduced pressure and subsequently crystallized as a pasty substance at cold temperatures (4°C) over a period of 30 hours. The recovered product was ultimately spray dried to yield a white powder. Difficulties were encountered however in the processing and removal of the final product by filtration after crystallization.

The Orafti process (Franck, 2002 a) on the other hand is divided into two phases. Phase one is carried out after the roots had been harvested and it involves the transportation and slicing of the chicory roots to produce “chips” from which inulin is extracted with hot water in a counter current diffuser (Figure 1.14). The effluent from the counter current diffuser is then treated with a primary purification step which results in impure syrups. The resulting leached chips are then dried and sold as feed.

Figure 1.14: Counter current diffuser used by Tongaat Hullet S.A in the processing of sugar cane. ©Hullet S.A. 2004

The primary purification step involves liming and carbonation of the chicory effluent at a high pH. The $CaCO_3$ sludge produced precipitates together with peptides, some anions, degraded proteins and colloids being trapped in the flocs. During this initial purification step, a foam-type product, rich in calcium and organic matter, is generated and is given to farmers for improving their soil structure (Franck, 2002 a).

Phase two of the Orafti process is concerned with the refinement of the chicory effluent using anionic and cationic ion exchange resins for demineralization purposes. After demineralization, the chicory effluent is sterilized by passing it through a 0.2 μm filter. Sterilization is then followed by evaporation and spray drying, and the final product is collected as a dry powder (Franck, 2002 a).

The Orafti process is however not without complications, as inulin purification procedures are considered to be compromised by balancing maximal removal of impurities against degradation of the inulin chain, colour formation, Maillard reaction (reaction between reducing sugars and proteins or amino acids which yields brown-coloured products and occurs naturally when certain foods are stored or heated), contamination and incorrect removal of taste and odour (Franck, 2002 a).

1.6.1 FOS production utilizing enzymes

In recent years the production of fructooligosaccharides, particularly those that range between GF_2 and GF_4, has seen an increase in the utilization of sucrose and fructosyltranferases (fructose transferring enzymes) derived from microorgannisms such as *Aspergillus* sp., *Bacillus* sp., and *Aureobasidium pullulans* (Park *et al*., 2001).

Fructosyltranferases are capable of producing fructooligosaccharides from sucrose through a consecutive set of disproportionation reactions (e.g. $GF_n + GF_n \rightarrow GF_{n-1} + GF_{n+1}$, n <4), while the remaining proportion is from $GF_n + GF \rightarrow GF_{n+1} + G$. The only disadvantage with this approach is the fact that fructooligosaccharides produced in this manner are subject to optimum reaction conditions such as temperature and pH which can alter the nature of the final product if slight deviations occur. Interestingly, crude, partially purified and completely purified enzymes have been observed to produce fructooligosaccharides of varying degrees, with GF_5 and GF_6 products mainly produced by crude fructosyltransferases while the smaller residues mainly GF_2 and GF_3 coming from the partially and completely purified enzymes (Park *et al*., 1999; Park *et al*., 2001).

1.6.2 Inulin characterization

High performance liquid chromatography (HPLC), with two columns in series in the K+ form (Aminex HPX-87 K+) is routinely employed for optimal separation of fructooligosaccharides to fructose, glucose, difructose-dianhydride (DFA), sucrose (GF), F2 and F3. However, further separation into DP3, DP4 and DP ≥ 5 is not accurate and as a result HPLC poses serious limitations in inulin analysis (Franck, 2002 a).

To circumvent the limited resolution observed with HPLC analysis, another technique known as high-pressure anion exchange chromatography (HPAEC) is successfully utilized to differentiate between GFn and Fn compounds, moreover, the method provides the molecular weight distribution of inulin (Figures 1.15 and 1.16).

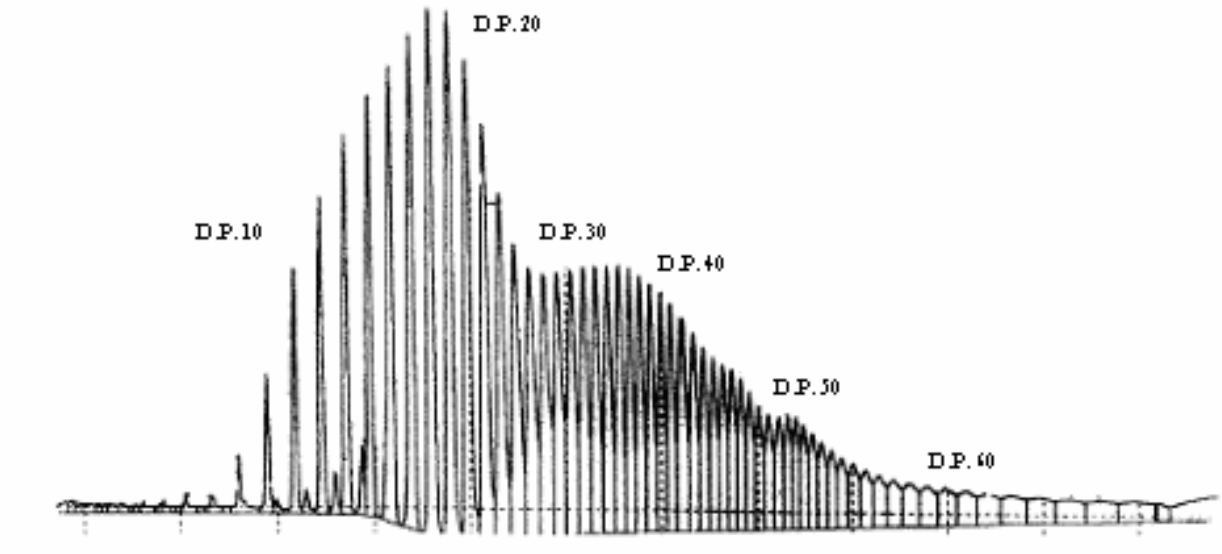

Figure 1.15 Dionex chromatogram of commercially available inulin (Sigma) from chicory. (Franck, 2002 a).

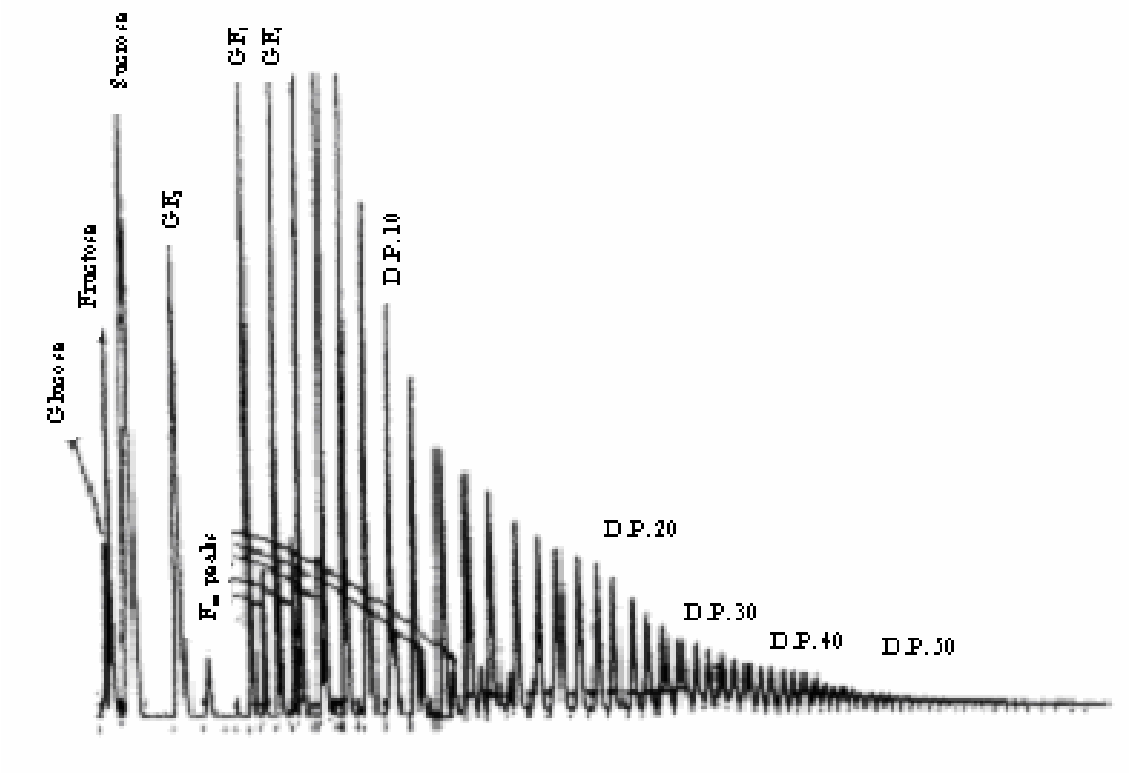

Figure 1.16 Dionex chromatogram of native inulin from chicory. (Franck, 2002 a).

HPAEC uses a Dionex series 4000 ion chromatograph (Carbo-Pac PA-1 column) that is usually coupled with a pulsed amperometric detector (PAD). Upon analysis, the carbohydrates are normally eluted with a NaOH/NaAc gradient; the hydroxyl groups are converted into oxy-anions by the high pH (13-14) of the NaOH. The carbohydrate retention time is then determined by the interaction of the oxy-anions with the anion exchange resin, while the PAD system oxidizes and detects the now separated carbohydrates as they pass through the detector (Franck, 2002 a).

The only drawbacks of HPAEC-PAD are the difficulties experienced in quantifying the high DP oligomers due to the lack of appropriate standards and the reduced sensitivity of the PAD detector for high DP polymers (Franck, 2002 a).

1.7 Aims and objectives

1.7.1 Aims

The aims of this study are to develop and optimize extraction and purification procedures for the industrial commercialization of inulin and fructooligosaccharides from chicory and to investigate the hydrolysis of the polymer using inulinases from the fungus *Aspergillus niger*.

1.7.2 Objectives

(i) To develop a simple and cost effective extraction of inulin and fructooligosaccharides from chicory (*Chicorium intybus*).

(ii) To develop an efficient purification processes of the inulin and fructooligosaccharides for the removal of colour, tannins and other contaminants.

(iii) To characterize the extracted inulin and oligofructosaccharides.

(iv) To extract and purify inulinase production from *Aspergillus niger* for subsequent inulin hydrolysis.

(v) To use inulinase in the hydrolysis of inulin to fructooligosaccharides.

Chapter 2

Inulin extraction

2.1 Introduction

2.1.1 Cell compartmentalization

As typical eukaryotes, plant cells are generally well compartmentalized, for efficient operation of processes like synthesis of organic molecules, energy transformation, growth and reproduction (Nadakavukaren and McCracken, 1985).

Even though most plants share a lot of characteristics, some differences in morphology that render the plants functionally different do exist. Vascular (chicory root) and supportive tissue plants possess secondary cell walls, predominant in cells that eventually provide support to the plant or conduct fluids, this is especially the case with xylem tissues, while some plants are only comprised of primary cell walls which consist of cellulose as the back bone of the cell wall's structural integrity. The cell wall thus provides the cell with mechanical stability preventing cell rupture as a result of tugor pressure and appropriate extensibility that allows cell expansion (Salisbury and Ross, 1992; Rieter, 2002).

Apart from other associated components such as structural proteins, cellulose, is the major strengthening component that serves as a structural molecule in plant cell walls. It is a linear polysaccharide that predominantly features 1,4-β-D-glycosidic residues assembled into paracrystalline microfibrils (each microfibril contains about 36 parallel polysaccharide chains). As structural entities, these polysaccharides permit the retention and transfer of information vital for the appropriate functioning of the cell (Rieter, 2002; Jensen, 1964).

Cell walls are present in a few different textures that ultimately determine the tensile strength of walls, of particular importance are the axial, transverse, crossed, helical, helicoidal and random, figure 2.1. The above mentioned textures then serve as the

prototype for the derivation of other textures. The manner in which cellulose microfibrils stiffen the plasma membrane, thus leading to a rigid cell wall, is governed by the orientation of cortical microtubules that lead to the transverse elongation of the direction of the cells (Emons and Mulder, 2000).

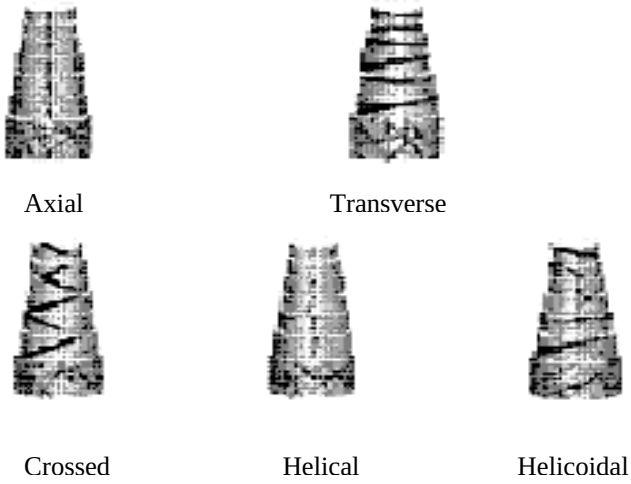

Figure 2.1: Cell wall textures; providing a closer look of how textures confer rigidity on cells (Emons and Mulder, 2000)

2.1.2 Cell to cell adherence

Cells do not exist as single entities, but in groups and adjacent each other. The middle lamella, region common to two cells, is rich in pectic substances (compounds composed of galacturonic acid) which function as a “cementing” material keeping cells intact. Apart from binding purposes they participate in plant defence through the release of signalling molecules or oligosaccharins (short sequences of 1,4- α -D -linked galacturonic acid residues) from the wall upon pathogenic attack (Nadakavukaren and McCracken, 1985; Pérez *et al*., 2000).

Thus, it follows that any interruption that disturbs the middle lamella so as to remove the pectin will result in cells falling apart. The cells will however, retain shape since they still possess other cell wall materials that furnish the cell with durability (Jensen, 1964). Figure 2.2 shows a group of cells and the middle lamella, where pectin is contained making it possible for cells to adhere to one another and keep their contents safely intact.

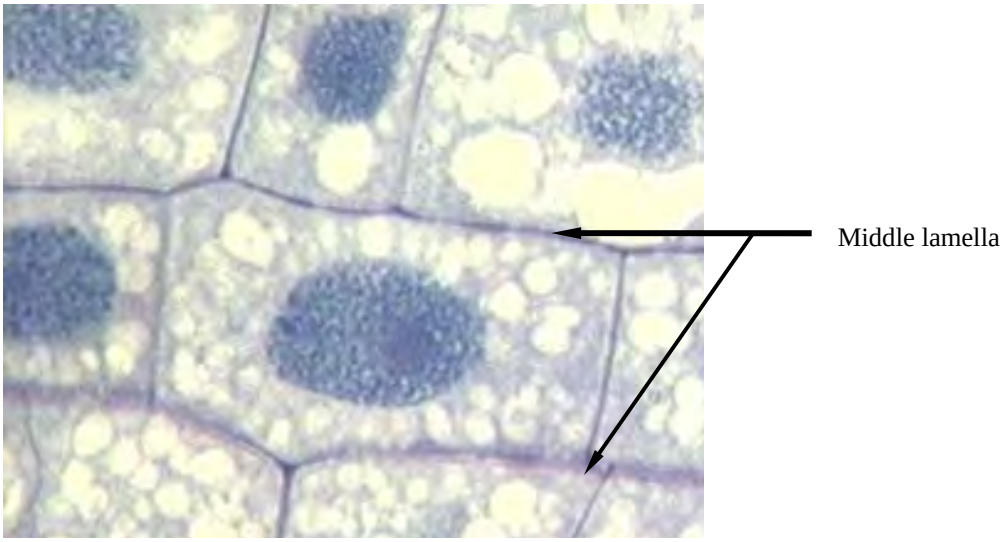

Figure 2.2: Location of the middle lamella ©Shaw, M. (1998)

2.1.3 Organelles within the cell

The cell is a multi-functional machinery that is continuously synthesizing metabolites that function in different processes. Different types of organelles involved in a multitude of transformations reside within the cell and has been repeatedly shown that these organelles are not independent in their function and that they supply each other with intermediate products for processing towards the benefit of the plant (Rieter, 2002).

The nucleus for example, is the control centre of eukaryotes and functions by determining the different kinds of micro and macromolecules that may be in need by the cell. In turn, these molecules influence chemical reactions that take place in the cytoplasm and the ultimate structures and functions of cells (Salisbury and Ross, 1992).

Many organelles function as storage bodies, for a variety of compounds that may be needed by the cell during stressful periods. For example, starch is particularly well distributed and is present in large quantities in almost all of the organs of higher plants. It is predominantly found as water insoluble granules confined to the plastids (Kruger, 1995).

2.1.4 The vacuole, inulin locality

Cells of a growing plant especially in roots and stems are normally occupied by abundant minute vacuoles. As cells mature the vacuoles constitute with each other while taking up water. Ultimately, a substantial amount of a fully grown plant's protoplasm is occupied by a large vacuole such that most of the cell's content is pressed against the cell wall by the large centrally positioned vacuole (Figure 2.3) (Raven, 1987; Jensen, 1964; Salisbury and Ross, 1992).

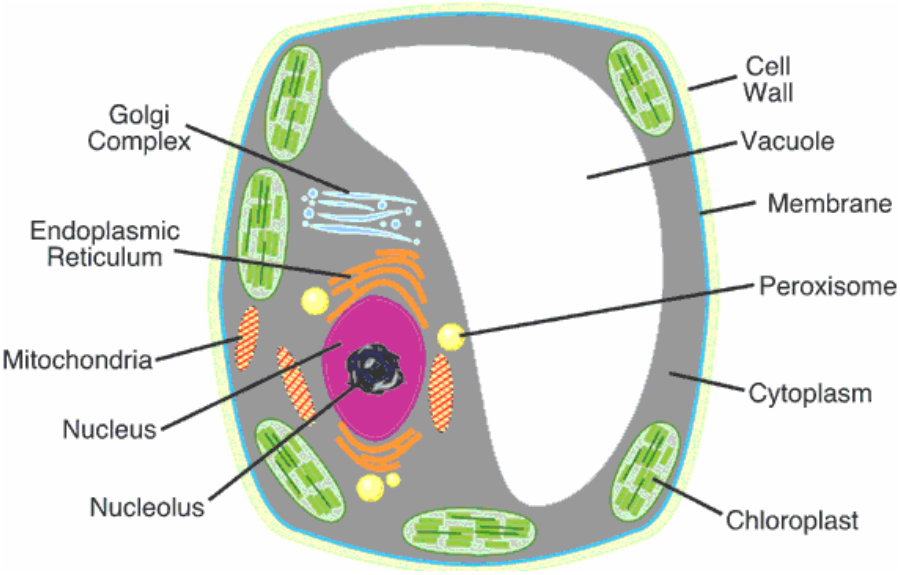

Figure 2.3: Plant cell with a conspicuous vacuole. The vacuole occupies much of the cytoplasm such that all of the plant's organelles are pushed against the cell wall. Armstrong (1989).

Vacuoles have the capability of storing large quantities of compounds that are vital for cellular metabolic processes without compromising the cell's osmotic pressure. Dissolved substances such as amino acids, proteins, sugars and pigments responsible for the colours of many flowers can be found within vacuoles. As a result during the plant's light phase, a general accumulation of photosynthetic products can be observed, and such metabolites are normally directed towards the vacuole for utilization during the dark phase (Raven, 1987; Salisbury and Ross, 1992).

Thus in fructan rich plant species, particularly chicory, inulin is almost entirely found in the vacuole along with free fructose and a very small proportion of glucose. The resultant accumulation of the fructopolymers start as a result of high photosynthesis activities in the chloroplast, where large amounts of carbon are generated and subsequently exported into the cytoplasm, resulting in increased sucrose synthesis. The sucrose is then pumped into the vacuole for inulin production through the action of fructosyltransferases and stored. A small proportion of unconverted sucrose is hydrolyzed into glucose and fructose by invertases (Figure 2.4) (Vijn and Smeekens, 1999).

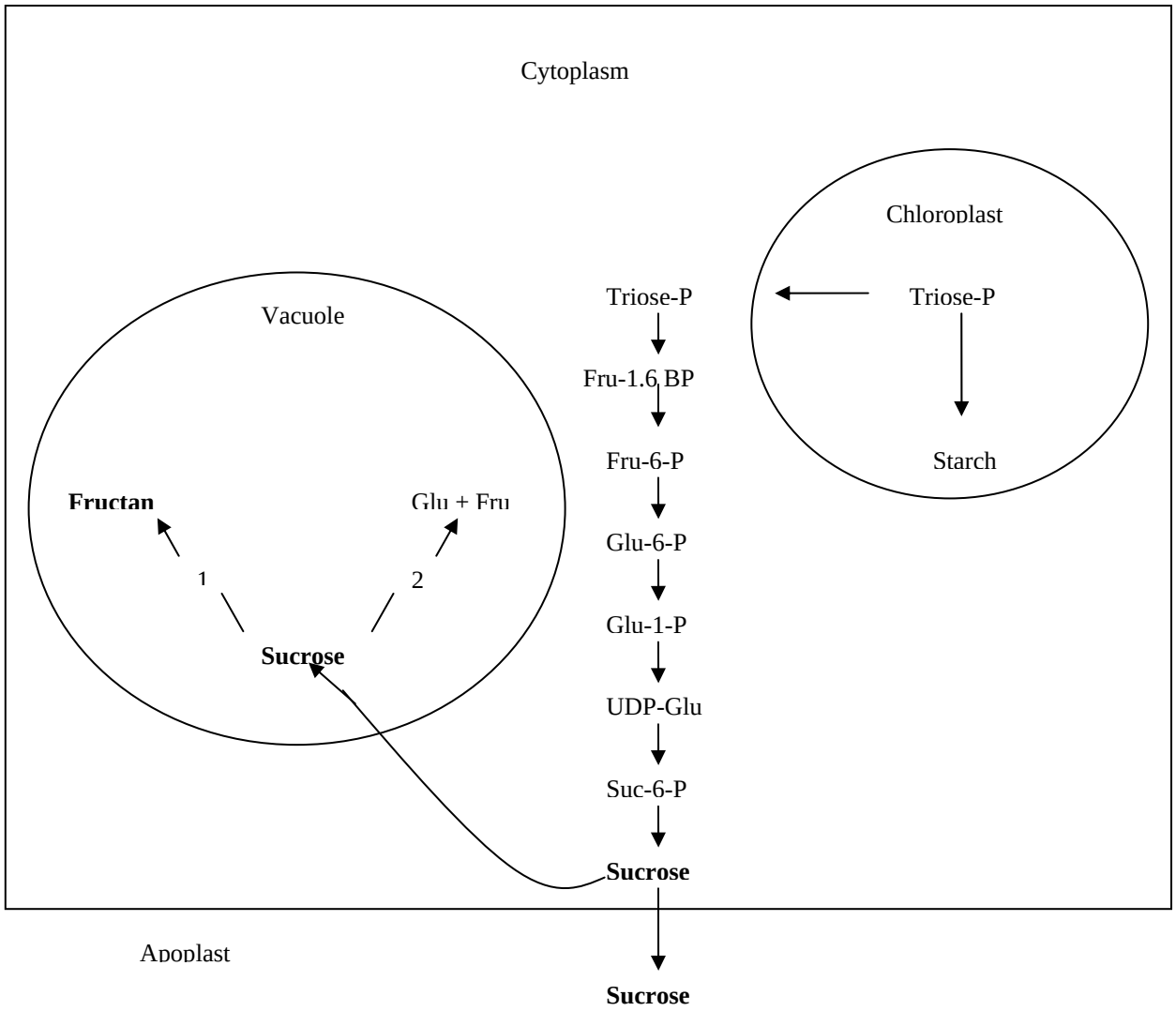

Figure 2.4: A schematic outline of fructan accumulation in the vacuole. Sucrose is synthesized in the cytoplasm through photosynthetically derived carbon. Sucrose is ultimately channelled into the vacuole, where inulin synthesis and storage takes place (1) and a negligible amount cleaved to glucose and fructose (2). Adapted from Vijn and Smeekens (1999).

2.1.5 Cell wall lysis

Figure 2.4 schematically depicts the inulin storage organelle. Unless, the use of enzymes (cellulases) is employed in cleaving the cell wall's cellulose responsible for the stiffened cell wall the cell will remain an enclosed entity that cannot be easily permeated. Chemical treatment of the cells is not a viable option as the target molecules' integrity might be compromised rendering the inulin unsuitable for human consumption. Cell wall disruption is further complicated by the presence of pectic substances that adhere cells to each other making the availability of every cell difficult. (Salisbury and Ross, 1992; Raven, 1987; Jensen, 1964).

Thus physical harsh techniques for cell disruption such as grinding and high temperatures (70 °C-80 °C) are required to ensure that the inulin is recovered without altering its state. Grinding disrupts the tough cell wall and the released pectin within the middle lamella is diluted, which allows the invasion of every cell through continued grinding. High temperatures enhance the isolation process as extraneous cellular components such as enzymes, inulinases within the chicory root, are inactivated, and inulin is very soluble (and not degraded) at such temperatures (Jensen, 1964; De Roover *et al.*, 1999; Henson, 1989).

2.1.6 Isolation of inulin from Dahlia tubers

Very few studies have focused on inulin extraction or alternative procedures thereof. Insufficient information or the use of expensive equipment is usually presented as seen in section 1.6 (the Orafti process, Franck, 2002 a). Despite the different extraction protocols, they all operate on the same principle (hot water extraction) and no radical deviations can be observed. The extraction process mentioned by Hébette *et al.*, (1998) in section 1.6 and indeed the one currently used by Orafti (Belgium) has not changed since Hirst *et al.*, (1950) succeeded in extracting (and methylating) inulin from dahlia tubers through a succession of steps that involved the mincing of tubers and expressing their juice through cheesecloth. After partial solidification of the juice hot water was added

followed by $Ca(OH)_2$ to elevate the pH to 8. The resulting precipitate was filtered off. The pH of the solution was lowered to 7 by the addition of oxalic acid, and cooled to 3°C which resulted in the separation of inulin. The inulin was kept in acetone overnight, filtered and dried. The supernatant was concentrated at 40°C and a further yield of inulin recovered. Consequently, the extraction protocol used in the current study was a modified version of the above.

The objectives of the study outlined in this chapter were to quantify the inulin content in chicory root and to develop and optimize extraction procedures for inulin and fructooligosaccharides.

2.2 Materials and Methods

2.2.1 Materials

All reagents used were of analytical grade. Chicory roots were supplied by Chicory S.A (Alexandria) after harvesting and were stored at –10 °C for later use. Commercial inulin (from Dahlia tubers), fructose, sodium potassium tartrate and sodium arsenate were purchased from Sigma (South Africa (Pty) Ltd). Ammoniun molybdate, copper sulphate, hydrochloric acid and sodium carbonate were supplied by Merck (South Africa (Pty) Ltd). A thermoreactor TR 420 (Merck South Africa (Pty) Ltd) was utilitized for the heating of the samples. For heating and stirring purposes, a hotplate magnetic stirrer supplied by Snijers (South Africa) was used. Roots were dried using a labotec TERM-O-MAT oven. Centrifugation was performed using a P Selecta ® mixtasel centrifuge. Solutions were concentrated using a Buchi rotavapor R-114 coupled to a Buchi waterbath R-480. Measurements of masses were accomplished through the use of a Denver instrument weighing balance. Absorbances were measured using Power Wave X (Bio-Tek Instrument Inc, South Africa) and helios Aquamate Thermospectronic (Merck South Africa (Pty) Ltd).

2.2.2 Methods

2.2.2.1 Determination of reducing sugars (Somogyi-Nelson)

The Somogyi-Nelson assay is based on the oxidation of sugars, with free anomeric carbons, by Cu^{2+}, and in turn the resulting cuprous oxide is reacted with arsenomolybdate to give a molybdenum blue colour which is then measured spectrophotometrically at 510 nm (Somogyi, 1952). The concentration of reducing sugars was estimated using a fructose standard curve (*Appendix 1*). Inulin contents of the raw extracts were measured as follows: inulin extract (0.1 g) was hydrolyzed by 0.8 M HCl (1 ml) at 90°C for 1 hour, after which the hydrolysate (150 µl) was tested for reducing sugars using the fructose standard curve. As inulin is insoluble in cold water an aliquot (150 µl) of the supernatant, obtained after suspension of inulin extract (0.1 g), was analysed for free fructose.

2.2.2.2 Preparation of the chicory extract

Chicory roots were washed, weighed and dried in an oven (60 °C, 24 hrs). The dried roots were then ground into a fine powder using a Waring commercial blender (New Hartford, Conn. USA). Distilled water was subsequently added to the powder (5:1 w/v).

2.2.2.3 HCl concentration for optimum inulin hydrolysis

In order to determine the amount of inulin from the chicory root powder and commercial inulin or isolated inulin, it was acid hydrolyzed into fructose with 0.8 M, which was then quantified by the Somogyi-Nelson method. Commercial inulin (100 mg) was added into each of the test tubes containing HCl (1 ml). The test tubes were heated in a thermoreactor (100 °C, 1 hour). At the end of this period, 100 µl of each of the hydrolysates were diluted (1:50) and 150 µl assayed for reducing sugars (Somogyi-Nelson assay).

2.2.2.3.1 Optimum temperature for inulin extraction

The chicory slurry (prepared in 2.2.2.2) was poured into five clean 100 ml beakers (5 ml in each beaker). The beakers were covered with parafilm to minimize evaporation and placed on hotplate magnetic stirrers. A temperature course study for maximum inulin release was then undertaken, where the beakers were heated while stirring at different temperatures (24-70 °C) for one hour. At the end of this period the chicory slurry was filtered through cheesecloth and the resultant solution centrifuged (9,000g, 30 minutes).

The pellets were discarded and the supernatants assayed for free fructose and inulin, measured as released fructose after acid hydrolysis. The hydrolysis reaction was carried out using 0.8 M HCl (1 ml) in tubes placed in a thermoreactor (90°C, 1hr) in order to measure the amount of inulin released at different temperatures. By subtracting the amount of initial free fructose from that produced by hydrolysis, the amount of inulin could be quantified (equation 1).

$$[I] = 162 \times [F]/180 \quad \text{(1)}$$

Where [I] is the inulin concentration and [F] the fructose concentration

2.2.2.3.2 Optimum heating period

To further optimize the extraction process, the same procedure as in section 2.2.2.3.1 was carried out at the optimum temperature and at varying periods (20 minutes to 3 hrs).

2.2.2.3.3 Inulin extraction

After cutting the root (207 g) into small pieces, distilled water (150 ml, 70 °C) was added and the root homogenized by blending for 1 minute. An additional 900 ml distilled water (70 °C) was added and heated (with continuous stirring) for 1 hour at 70 °C (the reaction vessel covered with parafilm to prevent excessive evaporation). The solution was filtered through cheesecloth and 0.1 M $Ca(OH)_2$ (70 ml) added to raise the pH to 8, and

subsequently lowered to pH 7 using 0.8 M HCl.. The pulp was re-suspended in 500 ml distilled water and the process repeated. This cycle required only 3.8 ml 0.1 M $Ca(OH)_2$ to raise the pH to 8 and returned to 7 utilizing 0.8 M HCl. Precipitates formed were filtered off and the solutions stored at -10 °C for 7 hours.

Upon thawing (section 2.2.2.3.3) two distinct layers could be observed, a pasty off white suspension and a dark brown top layer. These were separately concentrated using a rotavapor (40 °C), under reduced pressure, and collected as a dry powder after freeze drying. A total amount of 1050 ml distilled water was used for the extraction process. A comprehensive extraction process is shown in figure 2.5.

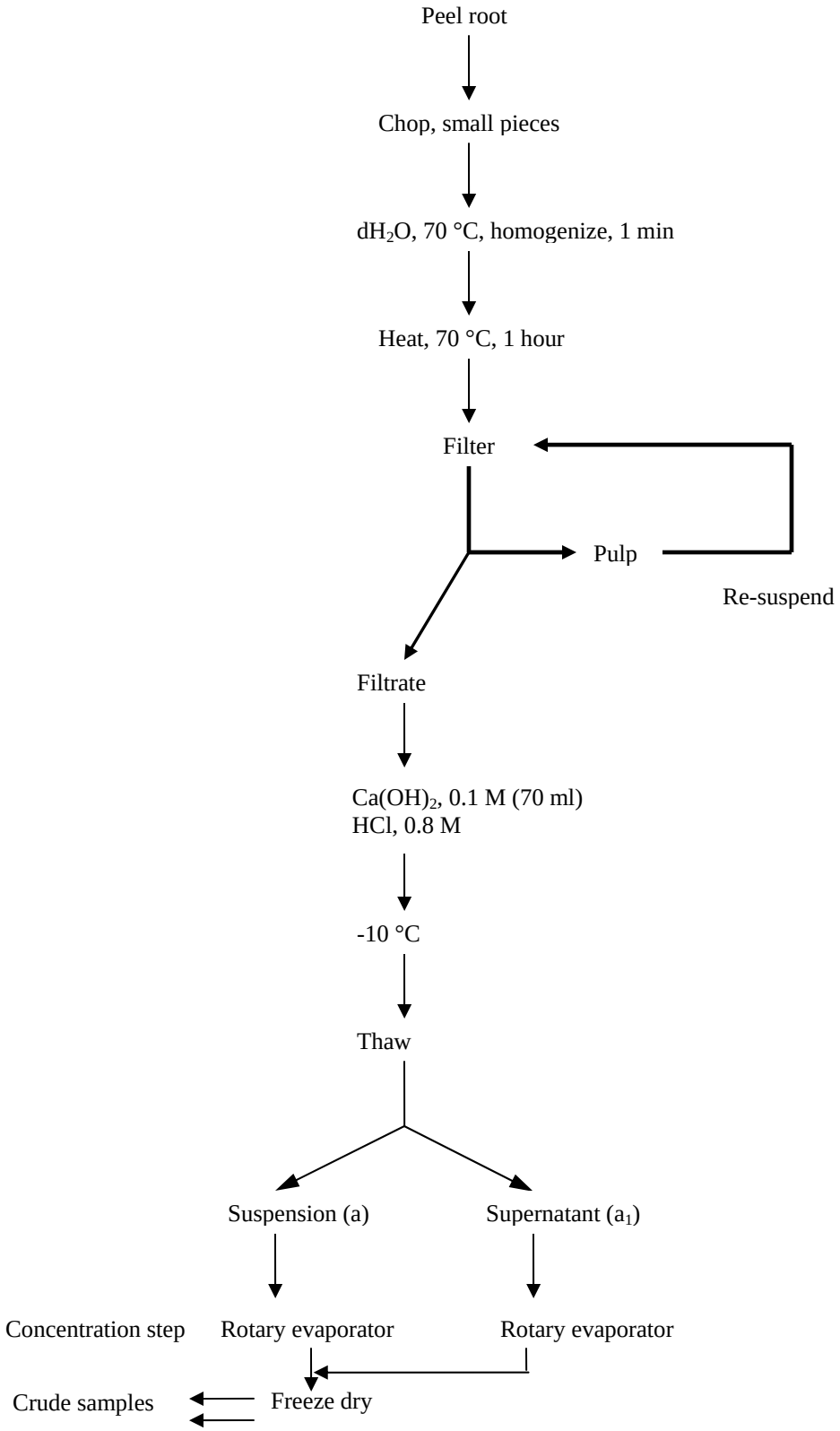

Figure 2.5: A flow chart of the raw inulin extraction process. The inulin was isolated from chicory roots using hot water and subsequently freeze dried and collected as powder.

2.3 Results and Discussion

2.3.1 Optimum HCl concentration

To find the optimum concentration of HCl for inulin hydrolysis, different HCl concentrations were tested on commercial inulin. The optimum acid concentration for hydrolysis was found to be 0.8 M. Figure 2.6 shows the optimum HCl concentration for the hydrolysis of commercial inulin. According to equation (1) (section 2.2.2.3.1), the total amount of free fructose released from commercial inulin (100 mg) should be 111 mg. In our process using 0.8 M HCl only 104 mg were produced (Figure 2.6) indicating a 93 % yield. Therefore, the method was deemed justifiable as there was not much disparity in recovered yields of fructose between the expected and the experimental results.

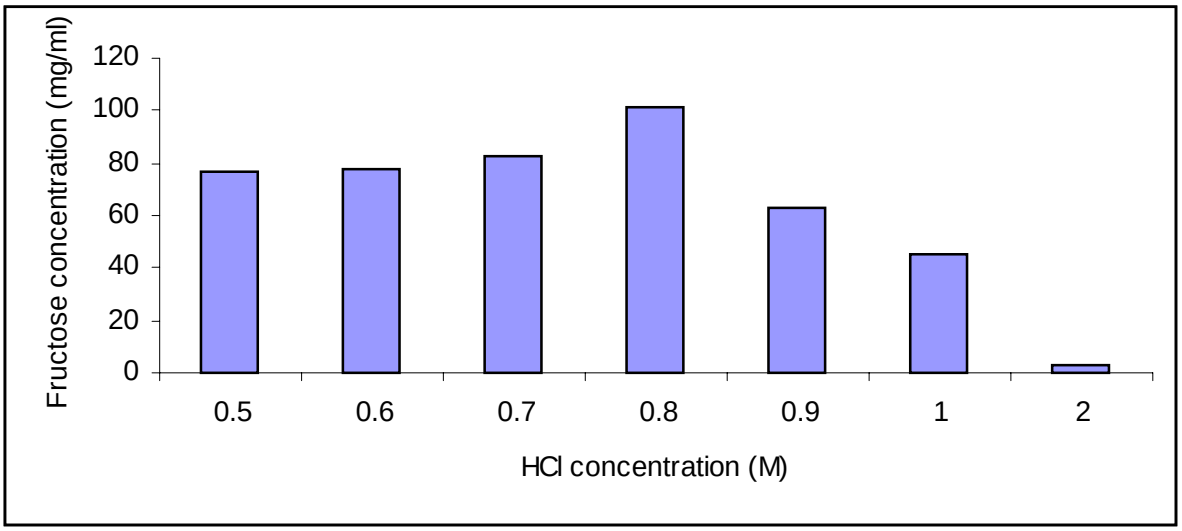

Figure 2.6: Optimum acid concentration for the hydrolysis of commercial inulin. The values are averages of duplicate experiments.

High acid strengths could have played a role in the degradation of fructose released from the polymer during the hydrolysis steps, HCl concentrations in the range of 10 M were observed to directly interfere with the Somogyi-Nelson assay. To prove this, a fructose standard curve was prepared (*Appendix 2*) and 50 µl (10 M) HCl added into each test

tube. The result was a zero detection of reducing sugars, which justified the claim that fructose released through hydrolysis was degraded and therefore not detected by the assay.

2.3.2 Optimum temperature and heating period

Preliminary studies using dried chicory powder showed inulin release from the root to be temperature dependent, while free fructose was readily available in equal amounts, irrespective of the temperature used. The ambient temperatures, 24 – 40 °C, were inefficient for maximum inulin release (Figure 2.7).

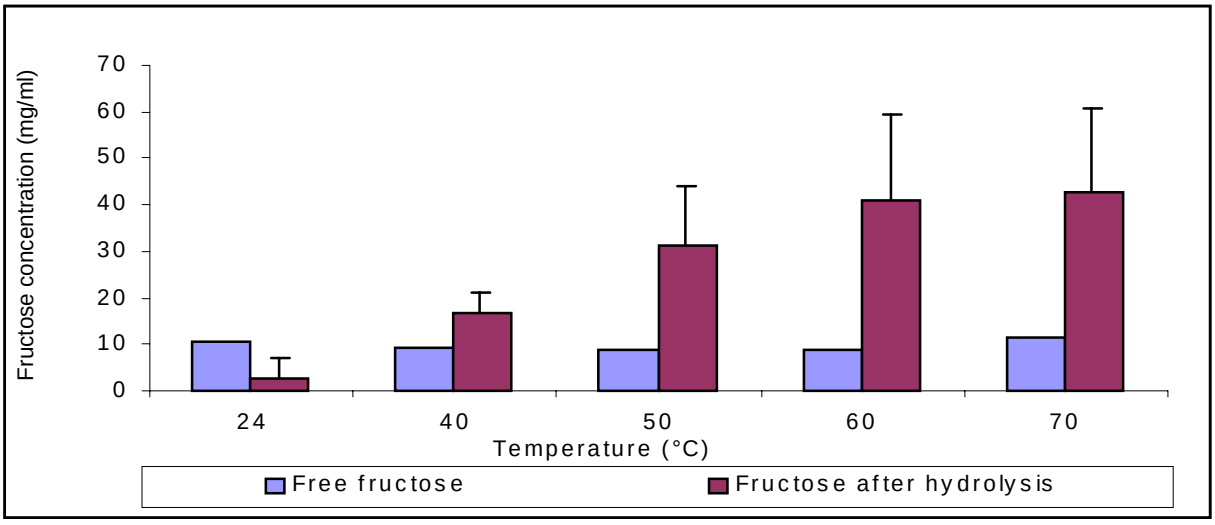

Figure 2.7: Optimum heating temperature for maximum inulin release. The values are averages of duplicate experiments. Vertical bars represent standard deviations.

Maximum inulin was released at 70 °C. At 80 °C the chicory slurry caramelized resulting in the absence of significant supernatant for assaying purposes. At 70 °C however, inulinases, particularly exohydrolase isoforms, 1-FEH I and 1-FEH II, which degrade inulin through the removal of terminal fructosyl residues, inherent in the root are denatured thus conserving the inulin chain. Even though chicory inulinase has optimum activity at around 35 °C, 1-FEH II still displays thermal stability at 45 °C, and only looses 80 % of its activity at 60 °C (De Roover *et al.*, 1999). This explains the concentration of free fructose at 24 °C.

To find the optimum heating period for the extraction process, the chicory slurry (prepared as above), was heated at 70 °C for different time periods. The slurry was filtered through cheesecloth and the procedure as in the determination of the optimum heating temperature repeated for assaying purposes.

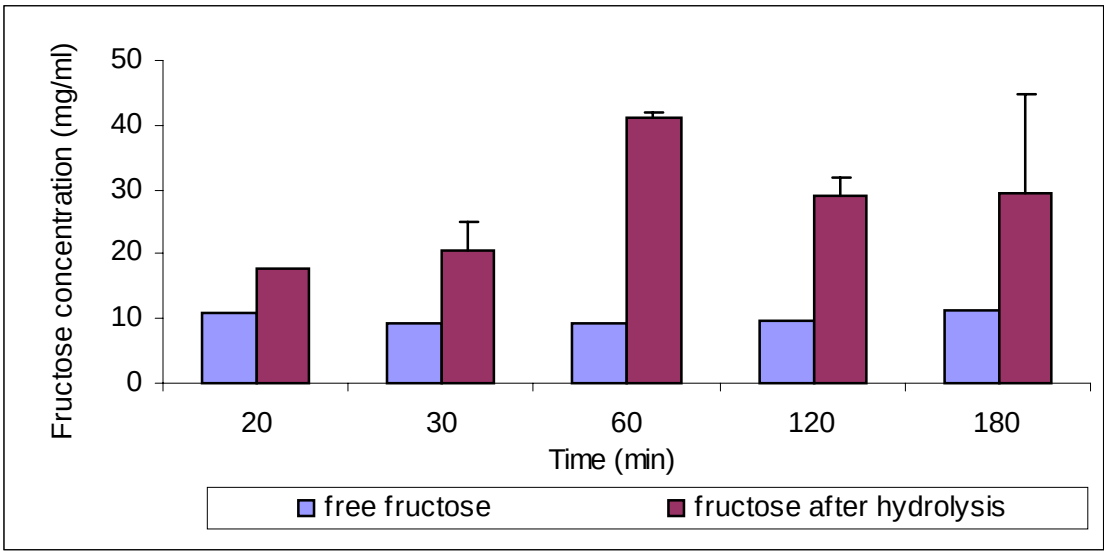

Figure 2.8: Optimum heating period in the extraction of inulin. The values are averages of duplicate experiments. Vertical bars represent standard deviations.

Heating at 70 °C for 1 hour resulted in maximum inulin release (Figure 2.8) and a consistent amount of free fructose. Consequently, all heating procedures involved in the extraction processes of the chicory homogenate were carried out at 70 °C for 1 hour, and a total of 1050 ml of distilled water was used for the homogenization of a 250 g chicory root.

2.3.3 Extracted inulin

Upon thawing (section 2.2.2.3.3) the two distinct layers, a pasty off white suspension — (a) contained 0.46 mg free fructose and 86.2 mg inulin, and a dark brown top layer — (a_1) contained 4.8 mg free fructose and 99.5 mg inulin. The percentage yields of the raw extracts were found to be 11 % inulin which was 80.2 % in purity and 4 % free fructose. When the amounts of inulin and free fructose, obtained from this study were compared with those obtained by Bubnik *et al*., (1997) where the chicory root contained, in dry

substances, 1.5 % free fructose and 16 % inulin. The extraction process was considered to be within an acceptable range in spite of the 5% inulin content difference, as the isolation method was cost effective and not as expensive and energy consuming as compared to the one used by Bubnik *et al*., (1997), which involved a four column countercurrent extractor where three columns ran concurrently while the change of cossettes ("chicory chips") ran simultaneously in the fourth one. During extraction, the columns were sequenced in series, and the countercurrent of the juice and cossettes was formed by the sequential automated switching of water inlet. An automatic refractometer equipped with through flow cell, continuously monitored the course of extraction.

Judging from the method used by Bubnik *et al*., (1997), it is apparent that the extraction method used in this study was convenient, less expensive and not as cumbersome, hence the acceptance of the 11 % yield.

2.3.4 Summary

Results obtained from this chapter suggest that for maximum inulin isolation, chicory roots must be processed as soon as possible after harvesting to prevent any inulin degradation (section 2.2.2.3.3). The observed amount of free fructose was more than that of inulin at 24 °C. The surplus of free fructose is probably due to the action of exohydrolases (mainly 1-FEH) which are generally induced by a drop in temperature (De Roover *et al*., 1999). Before inulin extraction, the roots had been stored in the freezer at -10 °C to prevent spoilage and subsequently thawed in preparation for extraction. It is probably during this period that the enzyme started cleaving the polymer thus resulting in a slightly pronounced free fructose concentration as compared to inulin.

Blending the root at 70 °C and subsequently heating the homogenate at the same temperature for 1 hour while stirring proved to be the most important step in the isolation of inulin. Moreover, 70 °C renders chicory exohydrolases inactive thus conserving the polymer from further degradation into fructose (De Roover *et al*., 1999). Freezing the heat treated homogenate was found to be crucial in the final recovery of inulin, as it is not

soluble in cold water and precipitates at low temperatures (Bemiller, 1972; Kim *et al*., 2001). Temperatures ranging between 5 °C and 24 °C did not give considerable amounts of inulin suspensions, hence, the use of -10 °C (which resulted in substantial yields). Optimization of the HCl concentration for hydrolysis purposes was vital for the quantification of inulin, as the unmodified acid (32 % HCl) affected the fructose standard curve resulting in undetectable amounts of fructose after hydrolysis.

The percentage yields of the isolate were found to contain 11 % inulin which was 80.2 % in purity and 4 % free fructose. These yields do not deviate much with those stated by Bubnik *et al*., (1997) where the chicory root contained, in dry substances, 1.5 % free fructose and 16 % inulin (amongst other components, such as water — 74 %, proteins — 0.3 %, lipids — 0.3 %, inorganic non-sugars and insoluble fibre — 1.3 %). Koch *et al*., (1999) on the other hand, found the chicory root to contain about 200g/kg of inulin, which is a much higher yield compared to yields obtained in this chapter. Moreover, the inulin extraction protocol used by Koch *et al*., (1999) is not mentioned making it difficult to exploit their extraction process in order to effect comparable inulin yields. As a result, the inulin yields obtained from this chapter can not be compared to those reported by Koch *et al*., (1999) for the assessment of an optimized inulin extraction process. Furthermore, given the fact that the extraction process undertaken in this chapter was meant to be a cheap and an economically viable process, compromises in low inulin yields could be accommodated, and the process was thus believed to be optimum.

Free fructose and inulin percentages given by Bubnik *et al*., (1997) and Koch *et al*., (1999) are not the norm with all chicory roots, since factors such as shelf storage and inherent inulinases can greatly affect the inulin and fructose distribution within roots (Bonnett *et al*., 1994). In light of the comparisons with Bubnik *et al*., (1997), the free fructose and inulin contents obtained from these studies suggest that the extraction procedure followed in section 2.2.2.3.3 was efficient in isolating maximum amounts of inulin. Moreover, the bulk of the free fructose was established to be in the top layer of the raw inulin extract. This identification was important in that a specific portion of the

extract can be exploited for the removal of these simple sugars, in the production of high inulin samples.

In its purified form inulin is recovered as an odourless white powder and oligofructose as a colourless syrup or white powder (Franck, 2002 b). The inulin samples obtained in section 2.2.2.3.3 were not free of impurities that contributed towards bitter principles and colour.

The respective phases of the raw inulin, the off white suspension and the brown supernatant, were found to contain significant amounts of tannins. The supernatant contained 30 mg/ml of tannins as opposed to the suspension which had 20 mg/ml. On concentrating the supernatant, in the process of inulin recovery, the tannins were simultaneously concentrated, thus contaminating the inulin and fructooligosaccharides, hence the brown colour and bitterness. The lowered concentration on the suspension probably explains the off white colour; nonetheless, it remained contaminated.

As a result Chapter 3 addresses the removal of contaminants such as tannins and the impacts of the purification process on the integrity of the final product.

Chapter 3

Inulin purification:

Batch and Continuous column chromatography

3.1 Introduction

3.1.1 Secondary metabolites

Plants are generally susceptible to attack by insects and foraging animals and as a result the need for defensive agents (secondary metabolites) arises. In many instances the production of secondary metabolites, which are toxic compounds that have no nutritional value to plants but benefit them by acting as deterrents to herbivores and insects, is not autotoxic to plants (Mckey, 1979). The defence systems extend to cover even microorganisms that manage to spread their infections from the locus of infection to peripheral regions of the plant. In such cases, the pathogenic products induce genes that are responsible for the plant's immunity resulting in systemic acquired resistance. For instance, in cases of fungal attack certain plant species release saponins which act by lysing fungal membranes thus sustaining the plants existence (Meneze and Jared, 2002; Woods, 2000).

Plant secondary metabolites are not only limited to defence and immunity but contribute in plant reproduction by attracting pollinators through the use of compounds that enhance the plant's ability to display attractive colours. Flavonoids, a diverse group of secondary metabolites, which include amongst others, flavonols, the most important of in the group, are particularly remarkable in this regard; they are ubiquitously synthesized by all plants, and apart from colour influences, they have been shown to play a role in protecting leaf cells against photo-oxidative damage (Menezes and Jared, 2002; Winkel-Shirley, 2002).

The reason why humans continue consuming certain plants is largely determined by a range of secondary metabolites that affect taste, colour and smell and they are probably

concentration dependant (i.e. occur in mild unharmful concentrations) (Verpoorte and Memelink, 2002).

3.1.1.1 Sesquiterpene lactones

The chicory plant has lavish green leaves which under normal circumstances should attract herbivores, and yet the root is always free from herbivore and insect attack. This observation is due to secondary metabolism. One of the major products of secondary metabolism in chicory are the terpenoids, predominantly the sesquiterpene lactones (lactucin, its *p*-hydroxyphenylacetic acid ester, lactupicrin, and 8-deoxylactucin) located in the leaf and latex of the root. As a result, fresh chicory has a bitter taste to humans (Rees and Harborne, 1985; Kisiel and Zelińska, 2001).

3.1.1.2 Tannins

Simple phenolics which are products of secondary metabolism play a major role in the production of tannins (polyphenols) through condensation reactions. Tannins have a variety of chemical structures, and are thus divided into hydrolysable (galloyl and hexahydroxydiphenoyl esters and their derivatives) and proanthocyanidins (PA), known as condensed tannins. The most detrimental effects that tannins have, is their ability to form insoluble complexes with digestive enzymes and dietary proteins (Chavan *et al*., 2001; Aerts *et al*., 1999). Chicory stores large amounts of condensed tannins in the vacuole. Precursors synthesized in the cytoplasm through a series of enzymatic reactions are channelled into the vacuole for conversion into tannins and storage (Aerts *et al*., 1999; Martin and Martin, 1982).

The presence of pectic polysaccharides and inorganic non-sugars is noted as vital targets in purification as their presence directly affects the purity of the final product (Pérez *et al*., 2000; Bubnik *et al*., 1997). Table 3.1 illustrates some of the secondary constituents found in the chicory plant.

Table 3.1: A summary of some of the secondary metabolites found in chicory. (Rees and Harborne, 1985).

Sesquiterpene lactones	Coumarins
1. Lactucin	10. Aesculetin
2. Lactupicrin	11. Cichoriin
3. 8-Deoxylactucin	12. Aesculin
Flavonoids	Caffeic acid derivatives
4. Luteolin 7-glucuronide	13. Caffeic acid
5. Quercetin 3-galactoside	14. Chicoric acid
6. Quercetin 3-glucoside	15. Chlorogenic acid
7. Kaempferol 3-glucoside	Tannins
8. Kaempferol 3-glucuronide	16. Proanthocyanidins
9. Isorhamnetin 3-glucuronide	17. Gallic acid

The three sesquiterpene lactones (lactucin, lactupicrin and 8-deoxylactucin) are ubiquitous in the roots and leaves; chicoric acid is only found in the roots while cichoriin and aesculin are predominantly constituted in the leaves; flavonoids are only present in the leaves.

As mentioned in section 3.1.1.2, these phenolics are present in the root and are water soluble (Chavan *et al.*, 2001). Thus, upon heating the chicory homogenate, a simultaneous diffusion of the secondary metabolites takes place resulting in contamination during concentration steps. Hence, the bitterness noticed with unpurified inulin (Franck, 2002 b) is carried forward.

3.1.1.3 Removal of tannins

Very few studies have focused on the removal of polyphenols from other plant constituents destined for human consumption. Nevertheless, polyphenols have been successfully extracted from plant material through the use of a combination of water and solvents. For instance, Jackson *et al*., (1996) managed to remove condensed tannins from the fresh leaves of *Lotus pedunculatus* by extracting with acetone/H_2O (70:30; v/v) containing ascorbic acid. The isolated extracts were then freeze dried and re-dissolved in a 1:1 solution of methanol/H_2O.

This method used by Jackson *et al*., (1996) is a standard procedure for the removal of tannins. Removing tannins in this way, especially when the compounds of interest are not polyphenols, tends to compromise the isolation and purification processes of inulin, in that solvents, such as methanol and acetone have a tendency to precipitate inulin and fructooligosaccharides. The precipitation of inulin is highly dependant on the ratio of solvent to water. For instance, at a ratio of 2:1 (v/v), only high molecular weight molecules are precipitated while the other molecules remain in solution. Whereas, a solvent ratio of 3:1 (v/v) simultaneously precipitates both high and low molecular weight fractions, while molecules made up of oligofructosaccharides remain in solution (Ku *et al*., 2003). Though this may appear to be a desired approach subsequent analysis of the product afforded inextractable compounds that failed to hydrolyse with HCl. Any follow up study on these precipitates were inconclusive and consequently new ideas for the removal of tannins (polyphenols) were sought.

Despite the challenges encountered in the exclusion of polyphenols from other plant components, Wall *et al*., (1969) succeeded in eliminating tannins by using, caffeine, lead acetate and polyamide (tested independently of one another). Consequently in the current study polyamide 6 powder was used as an effective agent in the removal of tannins.

3.1.1.4 Cationic and anionic ion exchange resins

Cationic and anionic ion exchange resins are currently being employed by the sugar cane industry for the demineralisation and "softening" of the sugar cane syrup (Hanine *et al*., 1992), which is usually followed by active carbon for decolourization. The disadvantage of using active carbon is the fact that the desorption of sugars especially, the monosaccharides and the dissacharides from the column requires the use of ethanol (5%) (Putman, 1950; Franck, 2002 a). To overcome such complications appropriate ion exchange resins such as Amberlite or Dowex have been identified by the sugar industry for successful preliminary purification processes. The advantage of using such resins is that, they are economical and can be used on their own for gross decolourization of highly coloured sugar solutions (Rohm and Haas, 2002).

Thus, the objectives of this chapter were to develop and optimize purification steps, using polyamide 6 powder (Wall *et al*., 1969), and ion exchange resins to demineralise and decolourize inulin solutions, thus removing the secondary metabolites. To achieve this, the feasibility of batch as opposed to column chromatography in the purification of inulin was assessed.

3.1.2 Materials and Methods

3.1.2.1 Materials

Polyamide 6 powder and Dowex® 4 were supplied by Fluka. Dowex® 50 WX 8 purchased from Serva. Amberlite IR-120 (Na) was purchased from British Drug House (BDH) and Amberlite type II ion exchange resin supplied by Rohm and Haas. $Ca(OH)_2$; HCl; NaOH; and Na_2CO_3 were of analytical grade. Magnetic stirrer by Snijders (South Africa). Absorbances were measured using either a Shimadzu visible 160A spectrophotometer or a helios aquamate thermospectronic (Merck South Africa (Pty) Ltd). CO_2 gas was supplied by Afrox. A peristaltic pump P-3 was supplied by Pharmacia Fine Chemicals.

3.1.2.2 Methods

3.1.2.2.1 Colour monitoring

In order to monitor the disappearance of colour during the purification of inulin, the λ_{max} of the brown colour was first determined by means of a spectrophotometric scan (200 nm to 800 nm) on the initial filtrate obtained from the filtered chicory slurry (section 2.2.2.2).

3.1.2.2.2 Tannin monitoring

The Folin-Dennis assay (Martin and Martin, 1982) was used to quantify the tannins and their concentration was monitored by a standard curve (*Appendix 3*).

3.1.2.2.3 Carbonation

All the purification steps were carried out before inulin crystallization. The bulk of the non sugars were removed through carbonation reactions (Vandamme and Derycke, 1983), where 0.1 M $Ca(OH)_2$ (70 ml) was added to the chicory homogenate (section 2.2.2.3.3), immediately after heating for 1 hour at 70 °C and CO_2 gas bubbled through the solution at different time intervals, from 0 to 50 minutes. The resulting precipitate was filtered off and the absorbance at 484 nm and pH of the supernatant taken at 10 minute intervals. This supernatant was monitored for tannins. As a control, the absorbance and pH of untreated chicory homogenate was measured.

3.1.2.2.4 Ion exchange: Batch purification

The chicory homogenate (section 2.2.2.2) was divided into 6 portions (50 ml each), and Dowex 50 WX 8 cation exchanger (previously regenerated and equilibrated in a minimum amount of distilled water) between 10 g and 60 g added to each beaker. The beakers were then stirred for 1 hour and the absorbances measured at 484 nm. The absorbance of the untreated homogenate was used as a control. The above procedure was repeated using Dowex 4 anion exchanger.

3.1.2.2.5 Polyamide 6

Polyamide 6 powder, (10g to 60g) was added into six different 100 ml beakers and immersed in distilled water. The distilled water was decanted (removing the fine particles) and chicory homogenate (30 ml) (section 2.2.2.2) added to each beaker and after stirring (1 hour) the colour and tannin concentration was measured. The chicory homogenate which had not been treated with polyamide 6 powder was assayed for tannins, and colour monitored to assess the efficiency of polyamide 6 powder.

3.1.2.3 Tandem (continuous) purification

3.1.2.3.1 Batch purification

Chicory homogenate (100 ml) was treated successively with carbonation ($Ca(OH)_2$ and CO_2 gas) according to section 3.1.2.2.3, filtered, then with polyamide 6 (60 g), filtered and ultimately treated with cation and anion exchangers (50 g) respectively.

3.1.2.3.2 Column purification

The cation exchange resin was regenerated by suspending in NaOH (100 ml; 0.5 M) and stirred for 30 minutes. The resin was allowed to settle, the NaOH decanted and washed with dH_2O (7 x). The resin was then re-suspended in HCl (100 ml; 0.5 M) and stirred for

30 minutes, after which, it was washed with dH_2O (7x) until the pH of the washings approached 7. The ion exchanger was subsequently put in a beaker and equilibrated with dH_2O. The method used for the re-generation of the cation exchanger was applied to re-generate the anion exchanger, except that HCl (100 ml; 0.5 M) was used in the initial step, followed by NaOH (100 ml; 0.5 M) and equilibrated with dH_2O as above, until the pH of all subsequent washings reached 7.

The ion exchange slurry equilibrated with dH_2O (above) was poured into a column and allowed to settle overnight under a flow of distilled water (1 ml/min)

The chicory homogenate in distilled water was passed successively into columns (1.6 × 30 cm) containing polyamide 6 column, Dowex® 50 WX 8 cation exchanger and a Dowex® 4 anion exchange (previously equilibrated in distilled water) at a flow rate of 1 ml/min. The eluent was collected and immediately frozen (section 2.2.2.3.3).

3.1.3 Results and Discussion

3.1.3.1 Spectrophotometric scan

The spectrophotometric scan (section 3.1.2.2.1) gave a peak at 484 nm, and this wavelength was then used to monitor colour disappearance during purification steps.

3.1.3.2 Carbonation

Variations in pH and colour disappearance with time during the carbonation step are shown in figure 3.1.

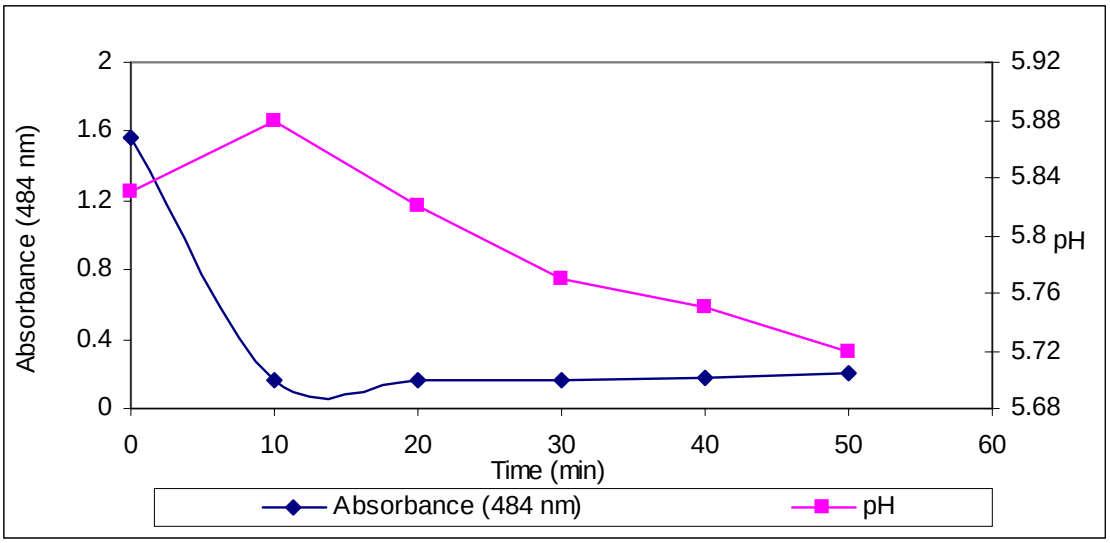

Figure 3.1: The effect of carbonation on chicory homogenate over a period of time with respect to pH variations (caused by CO_2 bubbling) and colour removal. The values are averages of duplicate experiments.

The pH of the chicory homogenate before $Ca(OH)_2$ addition was 5.83 with an absorbance of 1.6 at 484 nm. The addition of $Ca(OH)_2$ prior to CO_2 gas bubbling temporarily raised the homogenate's pH to 8 which immediately dropped to about 5.84. This effect was probably due to an increase in dissolved CO_2 as H_2CO_3 is formed.

After 10 minutes of bubbling CO_2 gas the absorbance of the homogenate at 484 nm dropped to 0.165 while the pH had increased to 5.88. The slight rise in pH was probably due to the $Ca(OH)_2$ that had not completely reacted with the chicoric acid. At this point a thick precipitate, presumably $CaCO_3$, could be observed, and the solution had lost a lot of colour. Bubbling CO_2 for more than 10 minutes did not remove any more of the colour yet there was a continuous drop in pH as the solution became more acid. Therefore, for all carbonation steps, CO_2 gas was bubbled through the solution for 10 minutes. This step managed to remove 90 % of the colour.

3.1.3.3 Ion exchange: Batch purification

Ion exchange resins were used to eliminate the tannins, terpenes and colour still contained in the chicory homogenate, as mentioned in section 2.3.4.

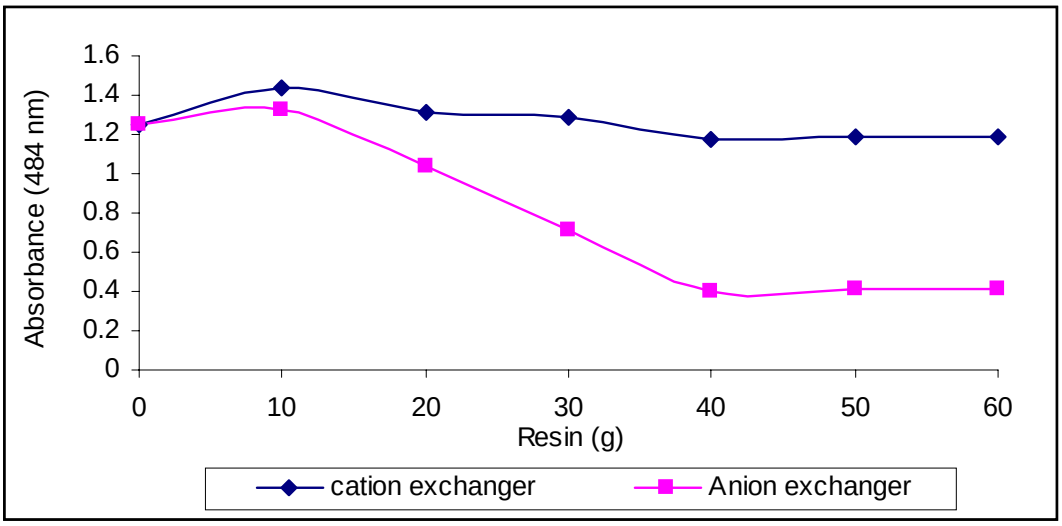

Figure 3.2: Shows the amount of ion exchange resin that is adequate for maximum colour removal. The values are averages of duplicate experiments.

The increase in absorbance observed between the untreated homogenate and the addition of 10 g of resin could not be explained. An increase in the amount of the ion exchanger used showed a concomitant decrease in colour especially where the anion exchanger was used, and 50 g of the anion exchange proved to be sufficient, for removal of 67 % of the colour (Figure 3.2).

The cation exchanger was not as efficient as the anion resin, even though it did remove some colour. However, this observation does not suggest that the use of the cation exchanger is unnecessary. Its importance is apparent for removal of salts (potassium, calcium, sodium and ammonium) of partial methyl esters of polygalacturonic acid that constitute the pectins (Lineback, 1999).

3.1.3.4 Polyamide 6

Polyamide 6 is a selective adsorbent that binds tannins irreversibly (Wall *et al*., 1969). Tannin removal by polyamide 6 proved to be weight dependant, 60 g polyamide 6 powder removed 92 % tannins as compared to 10 g which removed 53 % (Figure 3.3).

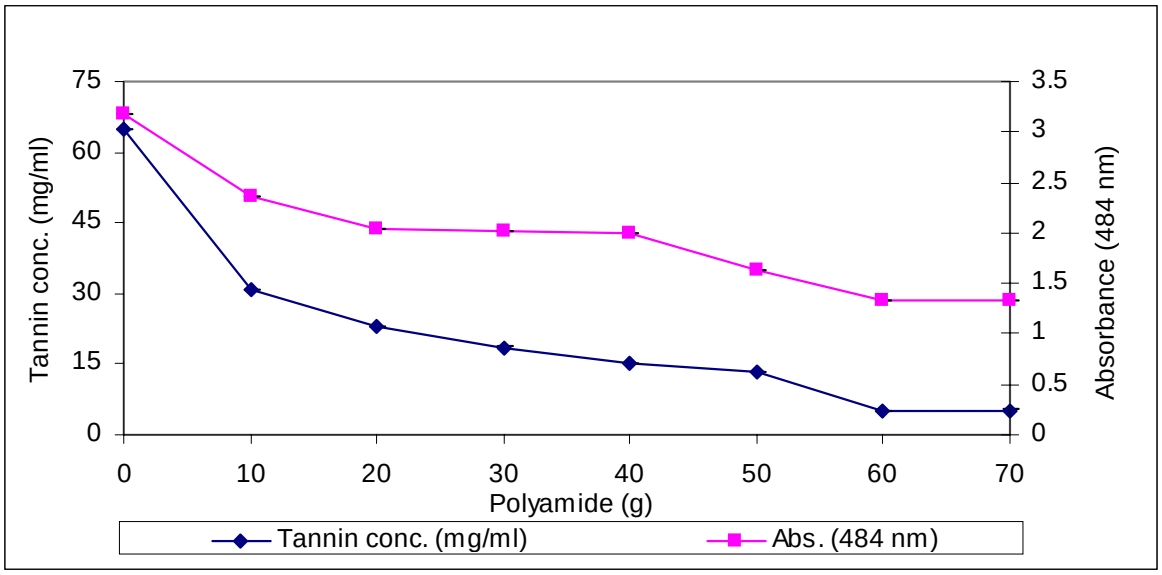

Figure 3.3: Polyamide 6 optimization and its effect on tannin concentration and discolouration. The values are averages of duplicate experiments.

Apart from tannin removal, polyamide 6 exhibited a tendency to remove colour as well, 60 g polyamide managed to remove 63.6 % of the colour as compared to 21.2 % observed with 10 g polyamide 6.

3.1.4 Tandem (continuous) process

The previous section (3.1.3) discussed discontinuous individual processes for purification. In order to explore commercial industrial extraction and purification attempts were now focussed on a continuous process.

3.1.4.1 Batch purification

Carbonation and polyamide 6 treatment of the chicory homogenate removed a considerable amount of tannins. There was still, however, a small amount of tannins and colour after the use of polyamide 6. This would be compromised in the final product, but was eventually removed by the two ion exchangers (Figure 3.4).

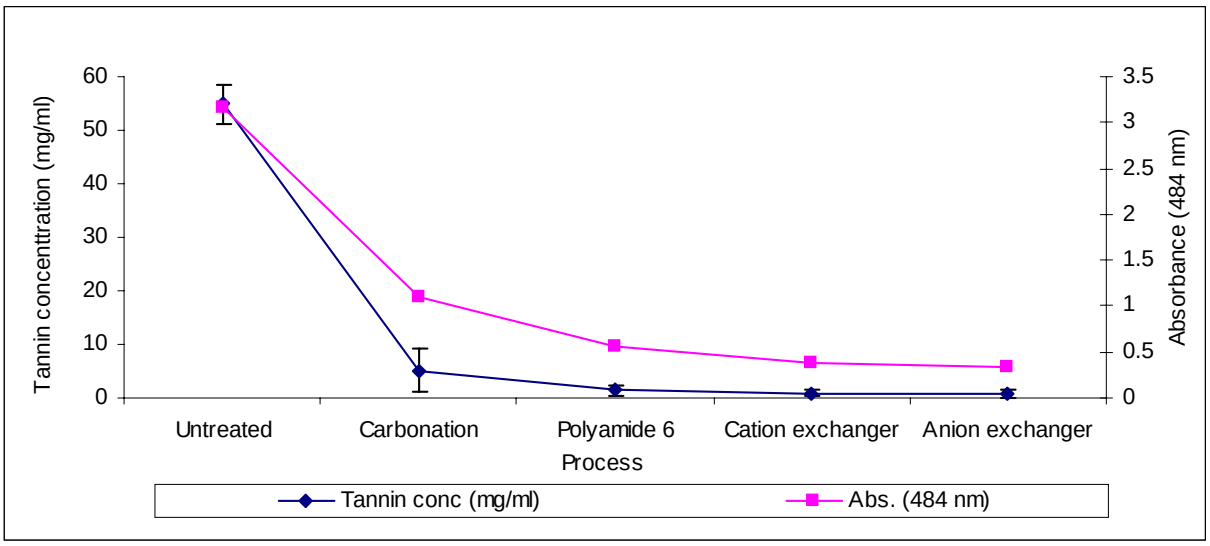

Figure 3.4: Discolouration and tannin removal by tandem continuous purification. The values are averages of duplicate experiments. Vertical bars represent standard deviations.

For efficient removal of colour and other non-sugars, carbonation was vital as a partial purification step. At the end of the purification process, colour and tannin concentration had decreased from 3.17 and 54.87 mg/ml to 0.33 and 0.75 mg/ml respectively. This decrease in colour and tannin concentration contributed to 89.6 % colour disappearance and 98.6 % tannin removal.

The amount of inulin obtained after each purification step (in figure 3.4 above) was found to be fairly constant (around 220 mg), while the quantity of free fructose dropped from 6 mg to 5.3 mg (Figure 3.5).

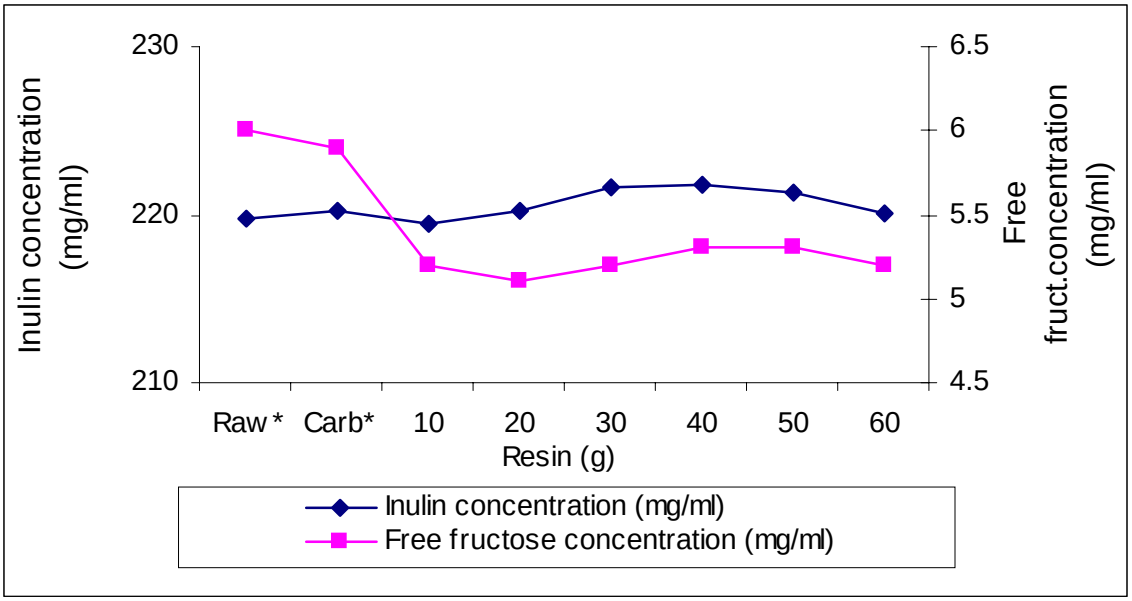

Figure 3.5: Monitoring inulin and free fructose contents during the purification process. The amounts of resin used refer to successive treatments with polyamide 6 powder, cation and anion exchange resins. The values obtained for each weight of resin were averaged (as there was no major deviation). Carb* = carbonation.

The drop in the amount of free fructose is directly related to the carbonation step. This could not be readily explained though the most probable cause could be attributed to the low pH of the solution due to carbonic acid formation which may have interfered with free fructose levels. This observation, is consistent with the one reported by Godshall *et al.*, 2002, where the amount of fructose following the clarification of sugar cane syrup with carbonation as the preliminary step, was found to have been completely destroyed, whereas glucose levels remained unchanged.

3.1.4.2 Column chromatography

After personal discussions with the technical manager at Chicory S.A it was anticipated that a continuous column chromatographic purification rather than batch treatment would be more advantageous. The above mentioned step for inulin purification was used in the development of a continuous system involving three columns (section 3.1.2.3.2) interconnected, and operated at 24 °C through the use of the peristaltic pump (Figure

3.6). The chicory homogenate (section 2.2.2.3.3) went through a carbonation step prior to being loaded onto the columns. Data for each individual step has been taken into account because of figure 3.5.

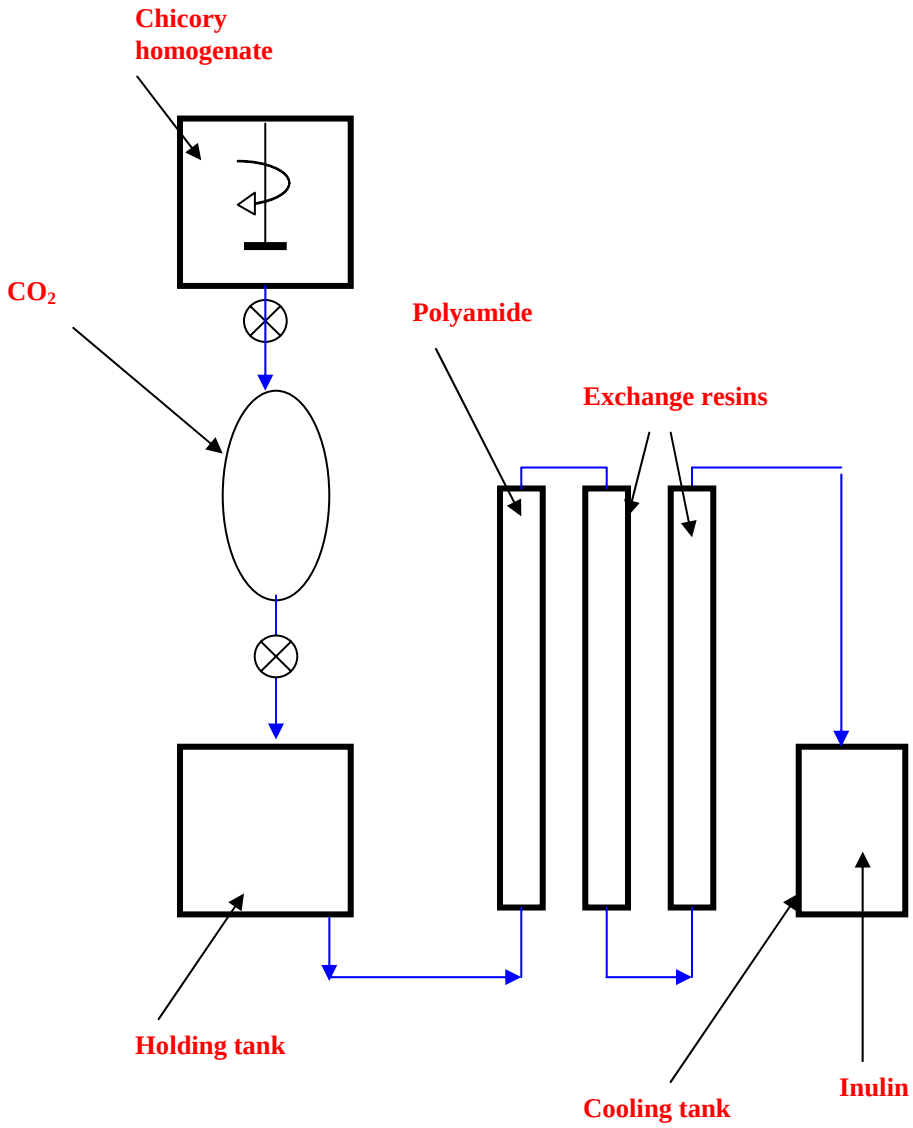

Figure 3.6: Continuous purification system for inulin.

After collecting the purified product (Figure 3.7 D) from the cooling tank (Figure 3.6), its analysis gave less than 1 % tannin and colour, 2.1 mg free fructose and 165 mg inulin, therefore was deemed to be pure enough for these studies. The different amounts of

inulin and free fructose obtained between the batch and the continuous purification systems are mainly due to different roots used.

From a technical point of view, however, the flow rate at 1 ml/min was unsatisfactory and any attempts to improve this were unsuccessful, hence the continuous column chromatographic process was abandoned.

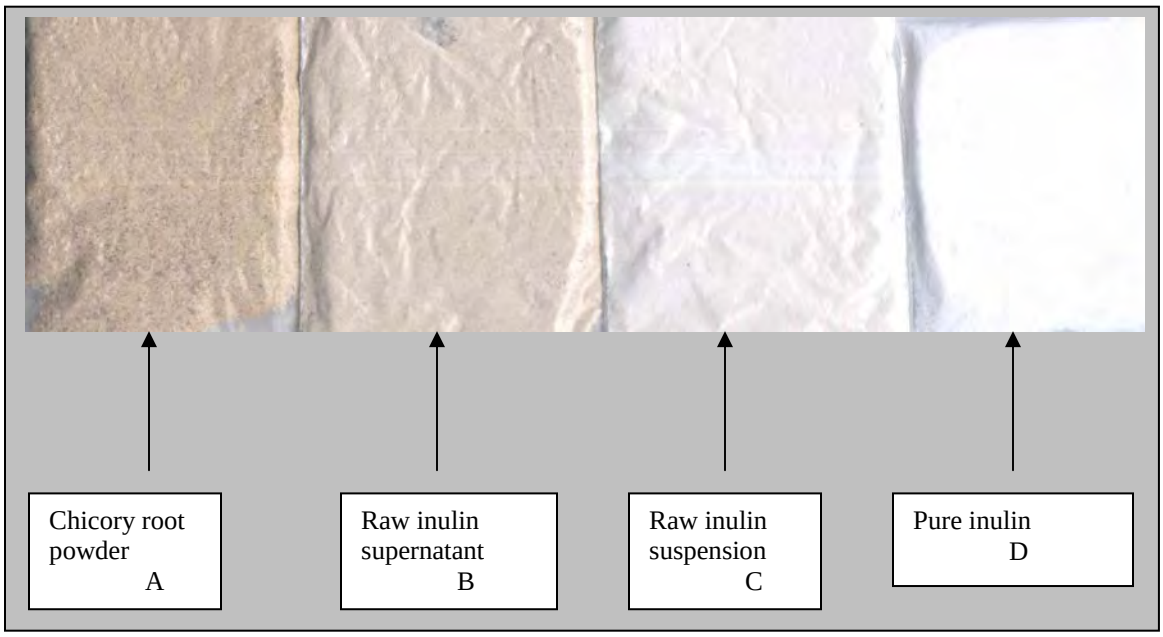

Figure 3.7: Raw and purified inulin samples. Sample A, is the chicory root powder on which ion exchange optimization for maximum colour and tannin removal was carried out. Samples B and C, are raw inulin products from the supernatant and suspension respectively (section 2.2.2.3.3). Application of tandem purification steps resulted in a pure inulin product (sample D).

Sample A (Figure 3.7) was the dried chicory root powder before inulin extraction and purification and as seen by the dark colouration, contained the non-sugars and tannins while samples B and C are the raw inulin samples (section 2.2.2.3.3), the difference in colour being due to tannin contamination. The difference in tannin contamination between the supernatant and the suspension of the raw inulin extract is due to the water solubility of these phenolic compounds, as mentioned in section 3.1.1.2. As a result, they accumulate in large amounts in the supernatant of the raw chicory extract. After purification samples B and C were subsequently combined into a separate pure sample, D. A comprehensive purification procedure is presented in figure 3.8.

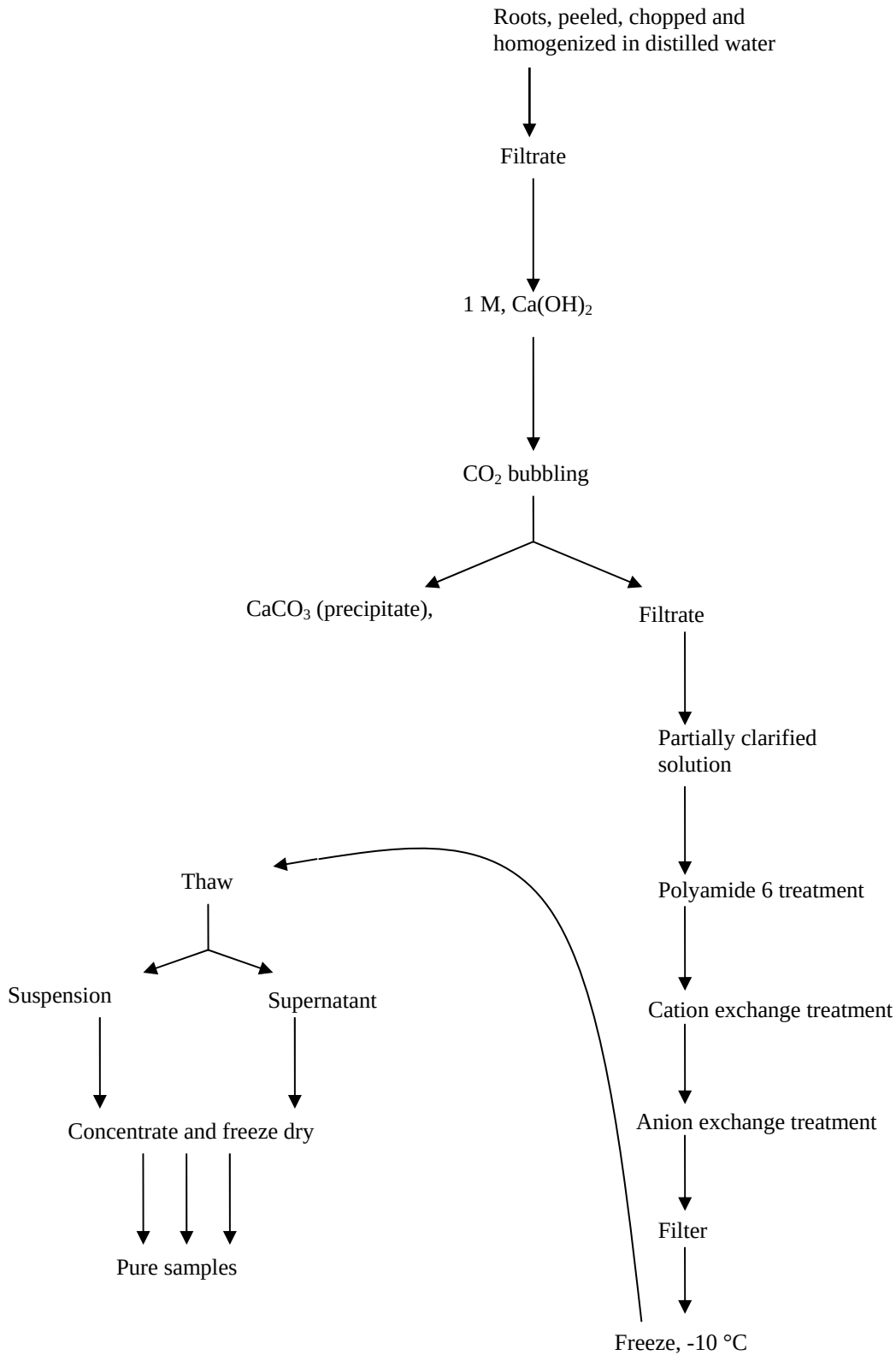

Figure 3.8: Purification flow chart.

3.1.5 Summary

For an efficient purification process, the chicory homogenate had to be processed immediately after preparation as there seemed to be fermentation and fungal growth when it was left unattended for more than 12 hours. Storing in cold temperatures as a preventative measure for fermentation, proved to be a counterproductive alternative, as the inulin from the chicory homogenate precipitated in its raw form.

For efficient purification of the chicory homogenate carbonation was an important step in that it removed most of the non sugars and colour which would not have been effectively removed by the other purification steps. As a preliminary decontaminating process, carbonation resulted in 90 % colour removal and 90.7 % tannin elimination. The ion exchange resins, particularly the anion exchanger, managed to remove 67 % of the colour from the chicory homogenate with slight tannin removal, whilst polyamide 6 powder removed 63.6 % of the colour and 92 % tannins. Subsequent linkage of the procedures for a continuous purification process resulted in 89 % colour and 98.6 % tannin removal.

As these studies were carried out towards the establishment of a commercial industrial process for inulin purification, factors such as high sugar recovery, product consistency and time management had to be considered. These factors would in turn determine the purification procedure to be adopted. In as far as sugar recovery and product consistency were concerned, batch and continuous column purification of the chicory homogenate were comparable and no major differences in inulin contents were noted, except where different roots were used. Tannins and colour were effectively removed by both processes.

The continuous column purification process operated efficiently at 1 ml/min and efforts to improve the flow rate were unsuccessful, and since one of the factors mentioned above (time management) was not fulfilled, the process was thus abandoned.

Polyamide 6 powder was effective in removing tannins and most of all, colour. It was not always possible, however, to regenerate it. In most instances it turned yellow and had a foul smell. Regeneration involved treating the powder consecutively with water, 50 % methanol, absolute methanol and 5 % acetic acid. (Wall *et al*., 1969).

At this stage the polymeric distribution of the pure inulin isolate was unknown, and therefore characterization procedures had to be undertaken. These differentiations are dealt with in the next chapter.

Chapter 4

Inulin and Fructooligosaccharide characterization

4.1 Introduction

4.1.1 Carbohydrate analysis

Carbohydrates are both the most abundant and structurally complex biopolymers found in nature. Proteins and nucleic acids are naturally available, however, they lack structural diversity, such as branched configurations, found in oligo and polymeric carbohydrates. For instance, simple hexasaccharides can exist in 1.05×10^{12} possible isomeric structures. This structural diversity creates difficulties in carbohydrate analysis, thus requiring more than one analytical technique for complete polymeric resolution (Harvey, 1999).

Various methods for the analysis of carbohydrates exist. These include high performance liquid chromatography (HPLC), high pressure anion exchange chromatography with pulsed amperometric detector (HPAEC-PAD), thin layer chromatography (TLC), gel electrophoresis (especially fluorophore-assisted carbohydrate electrophoresis, FACE), chromatographic techniques (gel filtration), mass spectrometry and static light scattering. Although powerful, these techniques have inherent draw backs that may compromise polymeric structural integrity, and can only be utilized in the analysis of a limited range of compounds. Moreover, some techniques have stringent requirements in that the analyte needs to be in its pure state before it can be analysed (Schiller and Arnold, 2000; O'Shea *et al*., 1998; Wolff *et al*., 2000).

Complex carbohydrate analysis has recently been simplified by adopting analytical techniques such as the matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF) which was primarily developed for molecules with large molecular weights, particularly proteins (Harvey, 1999).

4.1.2 Analytical techniques

HPLC is a reliable analytical procedure for the quantitative determination of sugars (mono-, di-, and some oligosaccharides) with degree of polymerization that range between 2 and 9. Thus, the analysis of complex carbohydrates generally results in poor resolution, as peaks tend to be superimposed, with resolution becoming less accurate from DP 4. Moreover, the refractive index detector (RI) that is used in most carbohydrate analyses suffers from a few drawbacks such as low sensitivity, lack of compatibility with gradient elutions, and reduced specificity. This is due to the fact that carbohydrate detection can only be achieved at low wavelengths where interferences and subsequent detection of non-carbohydrate compounds can occur. Consequently, this limitation implies that HPLC analysis of inulin ($2 \leq DP \leq 60$) and fructooligosaccharide mixtures is compromised and can not provide viable spectra (Coussement, 1999; Henshall, 1999).

Nevertheless, many complex carbohydrates have been successfully resolved utilizing HPAEC-PAD that provides informative spectra. HPAEC-PAD is routinely used in the quantitative analysis of inulin, and does not suffer from the major draw backs encountered with traditional HPLC, it is sensitive and does not require any derivatization of sugars (Hardy and Townsend, 1994; Henshall, 1999). Unfortunately in our case, such equipment was not readily available.

Alternative analysis using mass spectrometry is not a viable option either, as the technique is based on the ionization of molecules either by ejection of an electron or capture of a proton, thus resulting in compound fragmentation (due to energy generation) (Burrin, 1989). As a result, attempts to determine the molecular weight distribution of inulin mixtures is complicated by this technique, as the inulin would be fragmented and its true degree of polymerization lost. However, modifications of the classical mass spectroscopy that allow the analysis of polymers of high molecular weight and low volatility utilizing “soft ionizations” that result in minimal analyte degradation (MALDI TOF spectroscopy) have been adopted from other applications and are now being exploited in carbohydrate research (Schiller and Arnold, 2000). MALDI TOF

spectroscopy is a convenient analytical technique in that it is rapid and very sensitive (from the low picomolar to the attomolar range). Moreover, it can tolerate measurable amounts of impurities that are typical of most biological samples. Its overall spectral size distribution is comparable to that of HPAEC and it can be quantitatively interpreted (Schiller and Arnold, 2000; Stahl *et al*., 1997).

Gel filtration is a fractionation technique that has been widely practised for more than thirty years. Polysaccharides, proteins, nucleic acids and other biological molecules have been successfully purified and characterized using this technique. Its convenience and ease of use are based on the fact that molecules in solution are separated according to their size differences as they pass through a column packed with a filtration medium, consequently, small molecules diffuse into the gel, whereas, large molecules are not impeded and are therefore the first to elute (Figure 4.1) (Mathews and van Holde, 1990).

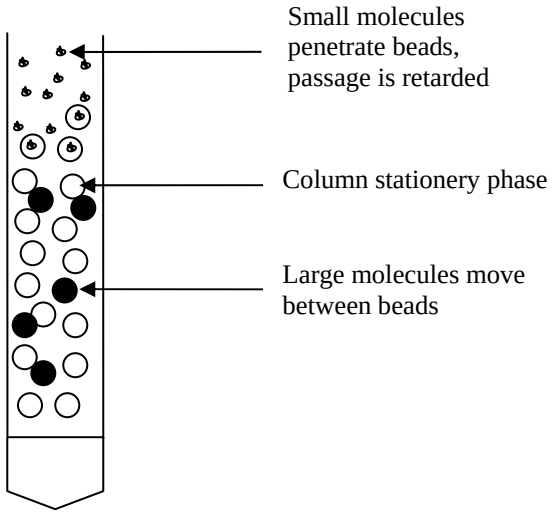

Figure 4.1: Diagrammatic representation of the principle of gel filtration. Mathews and van Holde, (1990)

Gel filtration is thus highly effective in separating heterogeneous samples with respect to molecular weight distribution for further quantitative analysis (Mathews and van Holde, 1990).

Polyacrylamide gel electrophoresis (PAGE) is the standard and ubiquitous method for the separation of large molecules such as proteins and for DNA sequencing. Recently, glycobiologists have been modifying and using the technique (FACE) for the separation of carbohydrates. However, complete and efficient separation of sugars requires that saccharides containing a reducing end be derivatized prior to electrophoresis (Jackson, 1994).

The principle behind this technique involves the reaction of a reducing terminus of a saccharide with a flourophore, 8-aminonaphthalene-1.3.6-trisulfonic acid (ANTS) (Figure 4.2) that contains a primary amino group. The carbonyl carbon then forms a Schiff base with the primary amino group and the bond formed is stabilized to a secondary amine using cyanoborohydride. Apart from conferring fluorescence, for detection, ANTS imparts a net negative charge, through its three sulfonic acid groups, that allows both acidic and neutral saccharides to be electrophoresed and consequently characterized using appropriate markers (O'Shea *et al*., 1998; Jackson, 1994).

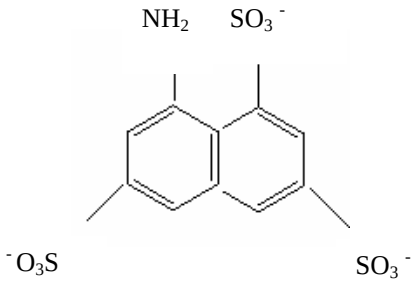

Figure 4.2: Structural formula of 8-aminonaphthalene-1.3.6-trisulfonate (ANTS). Jackson (1994)

4.1.3 Materials and Methods

4.1.3.1 Materials

TLC aluminium sheets (silica gel 60) were purchased from Merck (South Africa (Pty) Ltd). Cyanoborohydride, Dimethylsulphoxide (DMSO), anisaldehyde spray reagent, Sephadex G-50, α-cyclodextrin hydrate, γ-cyclodextrin hydrate, maltoheptaose hydrate, maltopentaose hydrate, maltotriose hydrate and stachyose tetrahydrate were purchased

from Sigma-Aldrich (South Africa (Pty) Ltd). ANTS was purchased from Merck (South Africa (Pty) Ltd)

4.1.3.2 Methods

4.1.3.2.1 Gel filtration on Sephadex G-50

Inulin purified in section 3.1.2.3 was a mixture of fructooligosaccharides and highly polymerized sugars. The sample was run on a Sephadex G-50 column for further application and development on the TLC plate. Purified inulin (0.5 g) was dissolved in dimethylsulphoxide (5 ml) and the sample applied onto a Sephadex G-50 column (1.4 × 63 cm). The column was eluted with distilled water and fractions collected (4 ml) at a flow rate of 0.17 ml/min. The fractions were assayed for free fructose and then hydrolyzed (section 2.2.2.3) for inulin quantification. Fructose was determined by the Somogyi-Nelson method (section 2.2.2.1) using a standard curve (*Appendix 1*). Fractions constituting respective peaks were pooled.

4.1.3.2.2 Gel electrophoresis of fluorophore labelled inulin

The inulin samples (1.5 ml) to be derivatized were dried in a microcentrifuge. ANTS (5 µl), prepared in 0.15 M acetic acid-water solution (3:17, v/v) and cyanoborohydride (5 µl, 1 M), prepared in DMSO, were added and the solution mixed. The mixtures were incubated for 16 hours (37 °C) and then dried in a centrifugal vacuum evaporator (CVE) for 1 hour and heated (35 °C) to vaporize the DMSO.

4.1.3.2.3 SDS PAGE

The molecular weights of the fluorophore labelled inulin were determined by SDS PAGE according to the method of Laemmli (1970). The inulin samples (10 µl), together with α-cyclodextrin hydrate, γ-cyclodextrin hydrate, maltoheptaose hydrate, maltopentaose hydrate, maltotriose hydrate, as molecular weight markers (10 µl) were electrophoresed

on 10 % SDS PAGE at a current of 15 mA (constant) for 1 hour, and increased to 30 mA (constant) for 2 hours. At the end of the electrophoresis the gels were viewed under UV light at 254 nm.

4.1.3.2.4 Thin layer chromatography

Sephadex G-50 fractions and reference mixtures (10 µl) of fructose, α-cyclodextrin hydrate, γ-cyclodextrin hydrate, maltoheptaose hydrate, maltopentaose hydrate and maltotriose hydrate were micropipetted onto TLC aluminium sheets (silica gel 60), and gently blown with a hair drier until the spots could not be seen. The plates were developed in two different consecutive glass tanks, the first containing n-butanol, acetic acid, H_2O, 2: 1: 1 and n-butanol, acetic acid, diethyl ether, H_2O, 9: 6: 4: 1, respectively for 1 hour in each tank. The plates were dried in a fume hood and incubated in an oven at 100 °C for 10 minutes. Migrated spots were detected by spraying the plates with anisaldehyde reagent.

4.1.3.2.5 MALDI TOF spectrometry

Inulin sample (10 µl) (from 1 mg/ml stock solution) was mixed with a saturated 10 µl MALDI TOF matrix (2.5-dihydroxybenzoic acid (DBH)) in 60 % acetonitrile/30 % water and 3 % triflouroacetic acid. The inulin-DBH mixture (2 µl) was then applied to a MALDI P100 gold sample plate and allowed to crystalize under a gentle stream of air. The plate was inserted (after confirming crystal formation by light microscopy) into a Perseptive Biosystems Voyager DE-PRO Biospectrometry Workstation that possessed Delayed Extraction Technology. Sample analysis was then carried out utilizing the preset method HCD1002 in the positive ion mode, which had an accelerating voltage of 20 kV, grid voltage of 94 %, Guide Wire Voltage of 0.05 %, delayed extraction at 400 ns and laser intensity of approximately 200 — 2500. Spectra were captured using Perseptive Grams/32 (R) v.4.14 level II.

4.1.3.3 Results and Discussion

4.1.3.3.1 Gel filtration on Sephadex G-50

The Sephadex G-50 chromatogram for the characterization of inulin is shown in figure 4.3 where peak 1 contained fractions 22 and 23, peak 2: fractions 24 and 25, peak 3: fractions 26 to 32, peak 4: fractions 33 to 37 and peak 5: fractions 39 and 40.

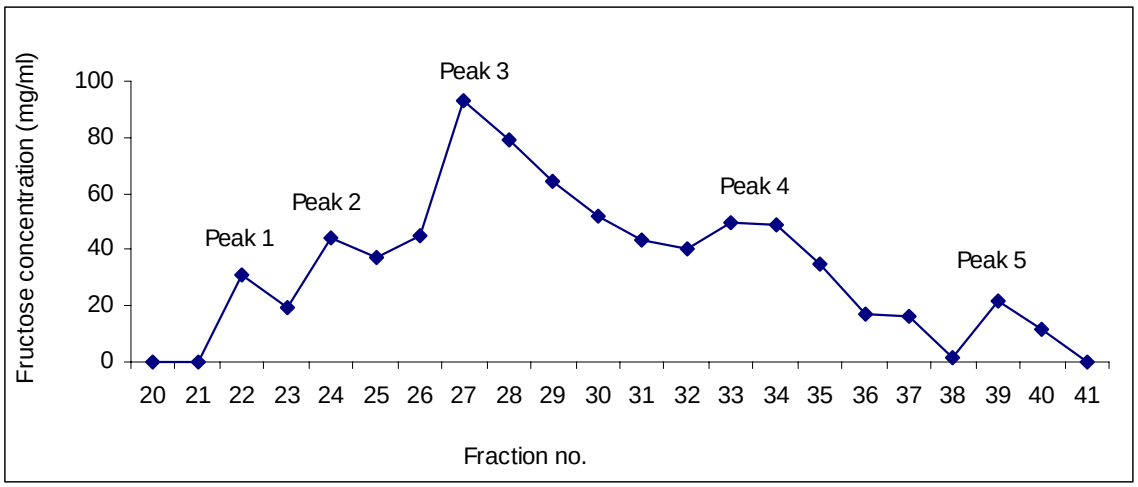

Figure 4.3: Sephadex G-50 chromatogram.

According to the principles of gel filtration the polymers of highest molecular mass would elute in the void volume and the components of low molecular mass retained within the gel and eluted later. Consequently, peaks 1 (approximately DP 27, 6.2 %) and 2 (approximately DP 18, 10.6 %) contained the most polymerized sugars (high molecular mass; highest DP), peak 3 (approximately DP 12, 57 %) a mixture of low and high DP fructooligosaccharides. Peak 4 (approximately DP 6, 22 %) contained the majority of fructooligosaccharides and less polymerized residues. Peak 5 (approximately DP 2-3, 4 %) contained low molecular weight units. The size exclusion chromatography was carried out as a preparatory step for thin layer chromatography.

4.1.3.3.2 Gel electrophoresis of fluorophore labelled inulin

No conclusive results were obtained as the technique was unsuccessful, despite attempts to increase the gel pore size from 8% to 16% in 2% increments. The samples appeared to have been trapped in the wells and did not move into the gel. This observation could not be explained, but it is possible that the inulin came out of solution during the electrophoresis.

4.1.3.3.3 Thin layer chromatography

Sephadex G-50 fractions (peaks 1 to 5) were applied onto a TLC aluminium sheet and developed as described in section 4.1.3.2.4 after both developing solvents (figure 4.4).

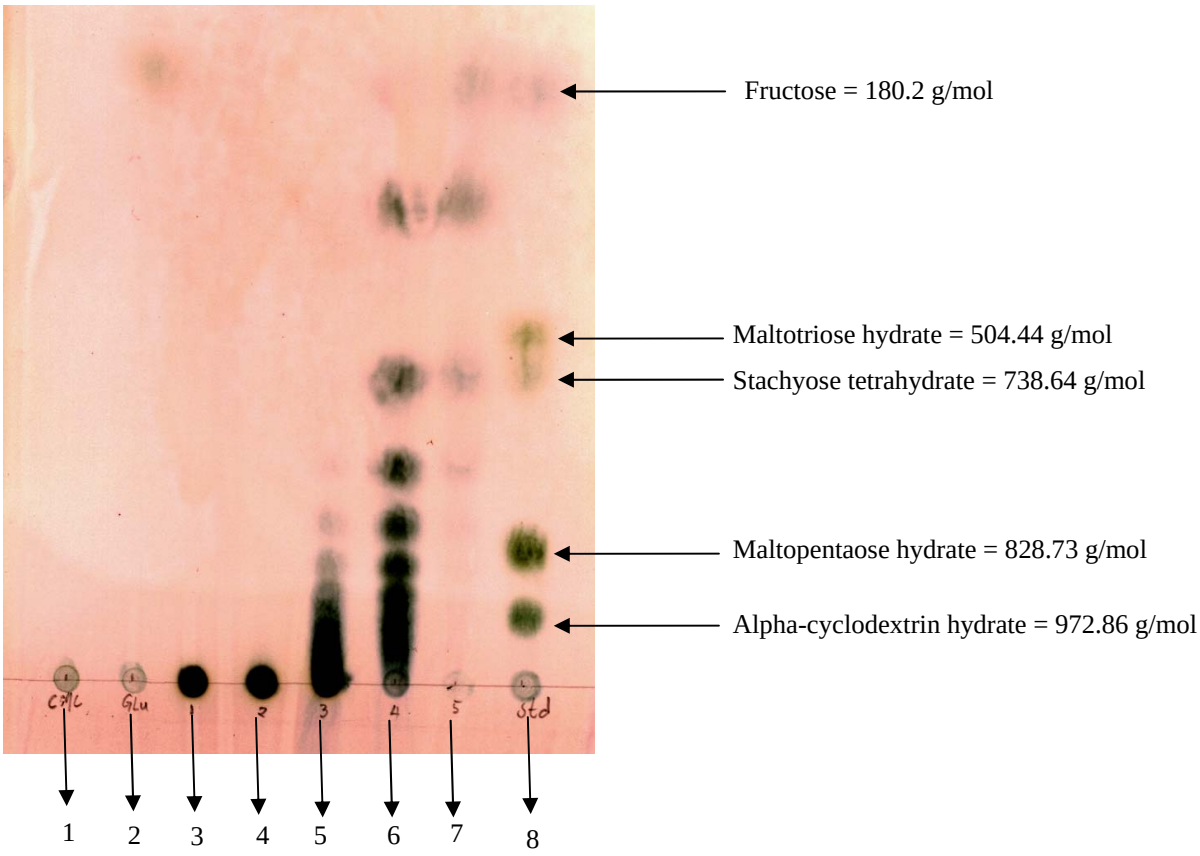

Figure 4.4: Developed TLC plate. 1 — carboxymethyl cellulose, 2 — glucose, 3 — peak 1, 4 — peak 2, 5 — peak 3, 6 — peak 4, 7 — peak 5, 8 — reference samples (fructose, α-cyclodextrin hydrate, γ-cyclodextrin hydrate, maltoheptaose hydrate, maltopentaose hydrate, maltotriose hydrate and stachyose tetrahydrate).

The reference samples used were as a result of a lack of appropriate inulin standards. The increasing molecular weights in lane 8 were used to approximate (given that the reference samples are substitutes) the degree of polymerization of the sugars separated on the Sephadex G-50 column.

Peaks 1 and 2 from Sephadex column (lanes 3 and 4 respectively) did not seem to move at all signifying that they were highly polymerized inulin components, while peaks 3 and 4 (lanes 5 and 6 respectively) were not adequately resolved. Lane 6 (peak 4), however, indicates that this fraction was probably made up of a mixture of fructooligosaccharides, as migrated spots translated into products of mass 180.2 g/mol and 972 g/mol. This suggests that peak 4 was largely composed of sugars that varied between single fructose residues, disaccharides (DP 2), trisaccharides (DP 3), tetrasaccharides (DP 4) and (DP 5) units. Lane 7 (peak 5) is indicative of low molecular weight sugars (fructose and disaccharides) with some tetrasaccharides (DP 4). The TLC analysis was not very conclusive with regards to the polymeric constitution of the purified inulin, and did not give a quantitative result, consequently a MALDI TOF analysis was embarked on.

4.1.3.3.4 MALDI TOF spectra

Figures 4.3 and 4.4 (MALDI TOF spectra) show molecular weight distributions of raw and purified inulin (DP conversion curve shown in *Appendix 4*).

The mass to charge ratio (m/z) obtained from MALDI TOF analysis of the raw extract, (figure 4.5) was found to range from 232.564 to 3607.96, thus representing a degree of polymerization that varied from 2 to 22 (DP conversion curve shown in *Appendix 4*). However, it was noted that this DP range was uncharacteristic of chicory inulin, as an anticipated maximum DP of around 60 was expected (Roberfroid, 2002). The 38 unaccounted fructose units could only be explained by the fact that the raw inulin sample was extracted from a root that had been defoliated and stored at -10 °C for a period of not less than one month. These treatments (cold and defoliation) probably induced chicory enzymes (chicory 1-FEH I and 1-FEH II) resulting in inulin breakdown (Ritsema and

Smeekens, 2003). As a different root was used for the extraction of purified inulin (figure 4.6), the DP was observed to range from 2 to 27.

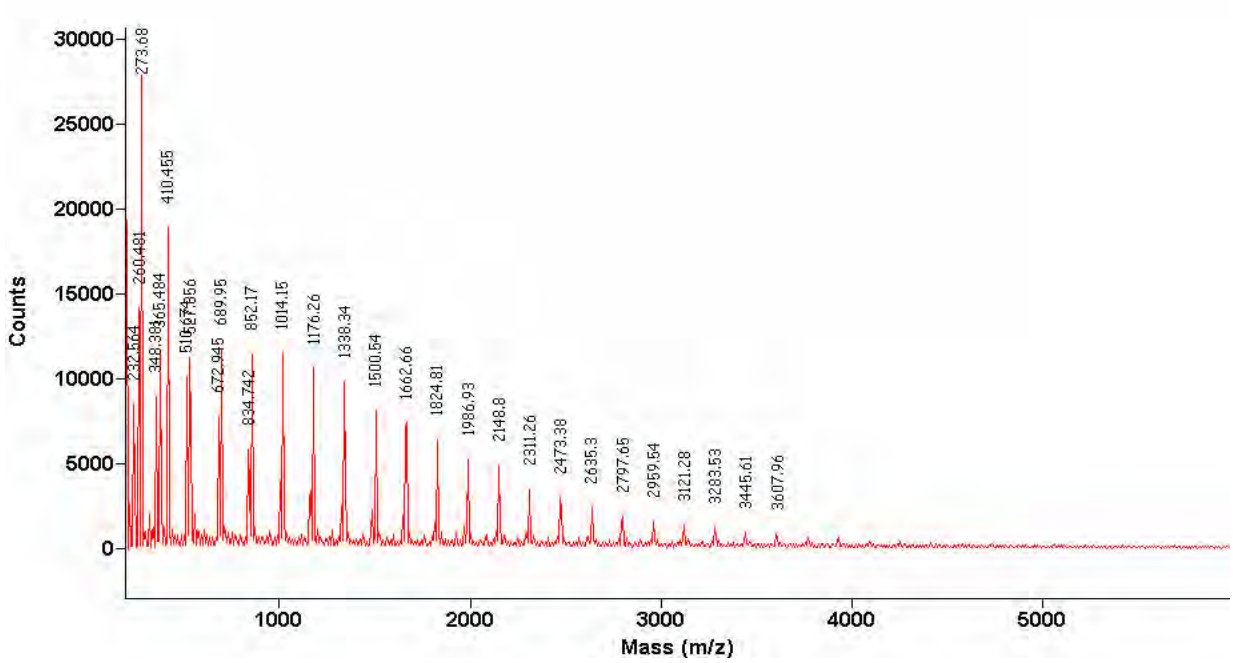

Figure: 4.5: MALDI TOF spectrum for raw inulin.

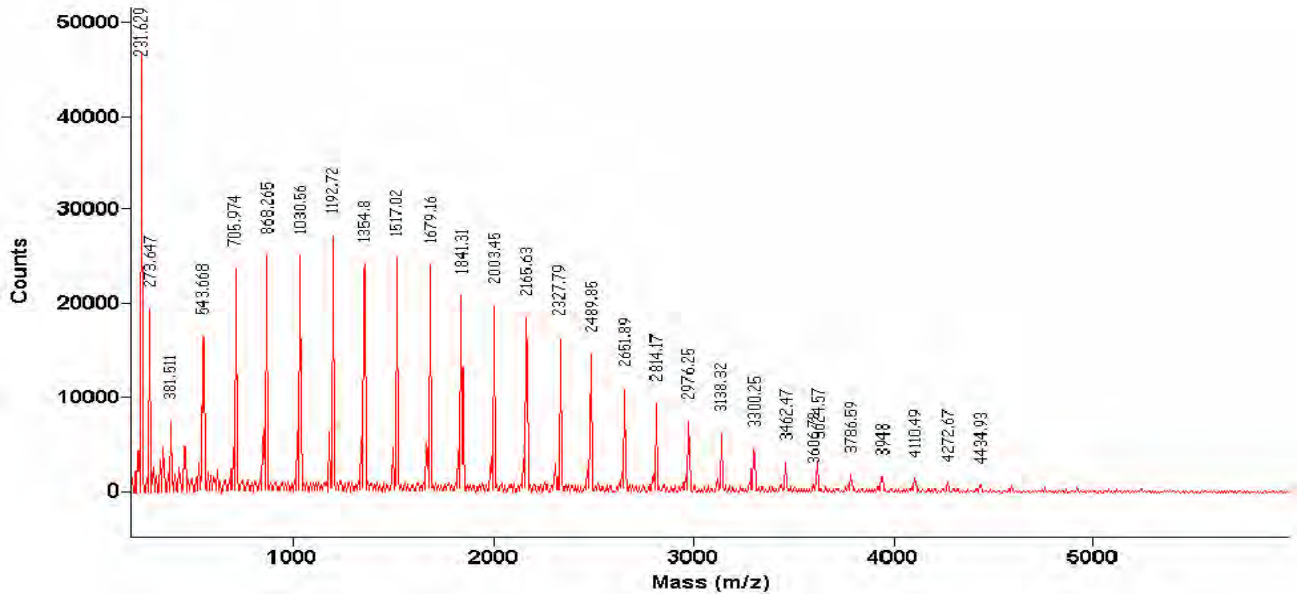

Figure 4.6: MALDI TOF spectrum for pure inulin.

Complete resolution of samples into respective masses by MALDI TOF is based on the subsequent excitement of the analyte into ions which are then accelerated in a strong

electric field, with typical voltages of 20 kV. As soon as the ions pass through a charged grid, they drift freely over a field free space. It is this field free pathway which influences sample differentiation. As a result, ions of low mass reach the detector faster than those of higher mass. The result is a molecular weight distribution spectrum (Figures 4.5 and 4.6) (Schiller and Arnold, 2000).

Due to the different speeds of the ions, it was deduced that fructose was the first to reach the detector. Therefore, the degree of polymerization was calculated by dividing mass to charge ratio by 162 ($MW_{fructose}$-MW_{water}). The first two peaks (232.564 and 231.629, figure 4.5; 4.6) were divided by 180, as these were unpolymerized moieties. The extent of polymerization observed for raw and pure inulin (section 4.1.3.3.4) was comparable to that found by Stahl *et al.,* 1997. Where maximum intensity for fructans isolated from *D. variabilis L*. was noted to be at an m/z value of 2635 which was equivalent to a DP of 16, which agreed with our findings, where raw and pure inulin (section 4.1.3.3.4) had a DP of 16 with an m/z value of 2635.3 and 2651.89 respectively. Peak intensities decreased with increasing size of the analyte molecules. Discrepancies in the distribution of degree of polymerization for inulin extracted in this study, where maximum DP for raw and pure inulin was 22 and 27 respectively could be attributed to enzymes inherent in the chicory root, as supported by evidence cited by Ritsema and Smeekens, 2003.

4.1.3.4 Summary

Sephadex G-50 was successful in fractionating the purified inulin sample into distinct components for further TLC analysis. TLC proved not to be appropriately quantitative for inulin analysis as high molecular weight components were not as adequately resolved as low molecular weight units (DP 2, 3, 4 and 5). Fluorophore-assisted carbohydrate electrophoresis of oligosaccharides was not successful, as no bands could be detected, even if the pore size was increased by varying the acrylamide concentration of the stacking and resolving gels.

In this study, MALDI TOF analysis proved to be a reliable and efficient technique for inulin characterization. Under normal circumstances analytical techniques that involve

ionization have stringent requirements for the removal of contaminating agents such as salts and buffers for reproducible analytical results. MALDI analysis, however, appeared to be less affected by the presence of contaminants, as observed with raw inulin.

The recovered inulin chain length was shorter than expected (DP 2 to 27) as opposed to DP 2 to 60, as cited by Franck (2002 b). Apart from the inherent chicory enzymes and cold storage, which ultimately lead to a shortened inulin chain, the date of harvest (35 weeks) could have influenced the observed depolymerization. At 35 weeks there is still active inulin synthesis from fructose (Luckman and Rossouw, 2003). Moreover, during the harvest period there is pronounced inulin depolymerization, which further shortens the polymer (Baert, 1997).

Chapter 5

Inulinases from *Aspergillus niger*

5.1 Introduction

5.1.1 Production of fructose syrups

As a sweetener, sucrose has been shown to have some negative health benefits such as carcinogenicity, contributes towards atherosclerosis and is contraindicatory to diabetics. As a result the sweetening industry has largely been focusing on the development of alternative substitutes (Vandamme and Derycke, 1983; Passador-Gurgel *et al*., 1996). Over the years fructose has emerged as a safe substitute that is void of sucrose related side effects, with inulin being the major source. Unfortunately, fructose recovery from inulin has always been hampered by inefficient production processes. It is a well known fact that oligofructoses can be produced by the acid hydrolysis of inulin, but this approach has several drawbacks, such as an undesirably yellow coloured hydrolysate (complicating purification procedures) and fructose anhydride formation which has no nutritional or sweetening value (Kim and Lim, 2002, Passador-Gurgel *et al*., 1996).

Conventional production of high fructose syrups involves the hydrolysis of starch (potato or corn) into glucose followed by D (+)-xylose isomerase treatment to produce a mixture containing 42 % fructose, 50 % glucose and 8 % other sugars. The resultant mixtures are called high fructose corn syrups (HFCS) (figure 5.1) (Vandamme and Derycke, 1983; Passador-Gurgel *et al*., 1996). HFCS preparations were subsequently followed by ultra high fructose glucose syrup (HFGS) production, using HFCS as a starting material. This process was efficient in delivering syrups containing more than 60 % fructose with glucose syrup collecting as a residue. To increase the fructose content the glucose syrups were subjected to an isomerase reactor (Vandamme and Derycke, 1983).

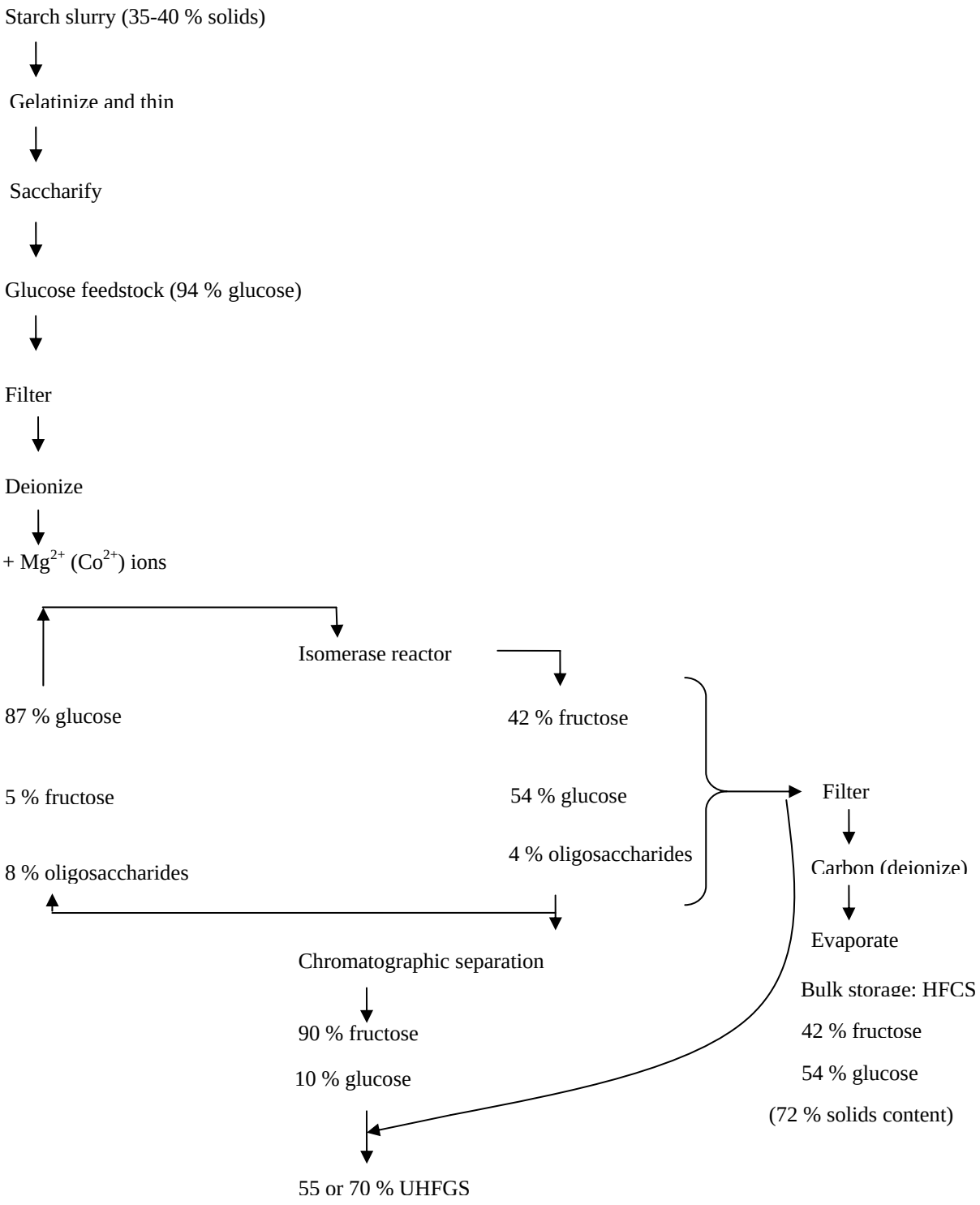

Figure 5.1: Flow chart describing the conventional production of fructose syrups. Adapted from Vandamme and Derycke, 1983.

5.1.2 Inulinases from *Aspergillus niger*

Inulinases are β-fructosidases that are utilized in inulin breakdown by releasing fructose moieties from the non reducing end of the β-2→1-glycosidic linkage by plants (exohydrolases, 1-FEH I and 1-FEH II) and microorganisms such as fungi (*Aspergillus niger sp, Aspergillus oryzae*, *Aspergillus ficuum* and *Aspergillus niger* 12) and yeasts (*Candida kfyr, Candida salmenticentis, Kluyveromyces fragilis* and *Debaromyces cantarellii*) (Vandamme and Derycke, 1983; Jing *et al*., 2002). The increasing interest in fructooligosaccharide production using enzyme hydrolysis is due to factors such as enzyme substrate specificity, controllable hydrolysis reactions that avoid the production of undesirable by-products, in comparison to chemical processing methods. Moreover, enzymes can be immobilized on solid supports for continuous processing and are biodegradable (MacCabe *et al*., 2002).

There are few similarities between plant and microbial inulinases, however, they are both exo-acting in their nature, with the exception of *Aspergillus niger* 12, *A. ficuum Penicillium purpurogenum* and *Chrysosporium pannorum*, which secrete both endo- and exoinulinases that act in synergy for efficient fructose recovery. The action of endoinulinase on inulin results in the production of inulotriose, inulotetraose and inulopentaose. The enzyme lacks invertase (enzyme catalyzing the hydrolysis of sucrose into glucose and fructose) activity as observed in most exoinulinases (Nakamura *et al*., 1995).

Biotechnologically, plant inulinases are not popular in that their production is seasonal and not inducible. Furthermore, microbial inulinases, especially those recovered from fungi, are easy to isolate as they are secreted extracellularly and the fungi can be manipulated to secrete them in large amounts, either genetically or by altering carbon sources (De Roover *et al*., 1999; Jing *et al*., 2002; Vandamme and Derycke, 1983; Nakamura *et al*., 1994). It has thus become evident that chemical hydrolysis of inulin is an unsustainable and undesirable approach as opposed to enzymatic methods.

Results from this section will be linked to the findings of previous chapters, for the development of a continuous inulin and enhanced FOS production process (figure 5.2).

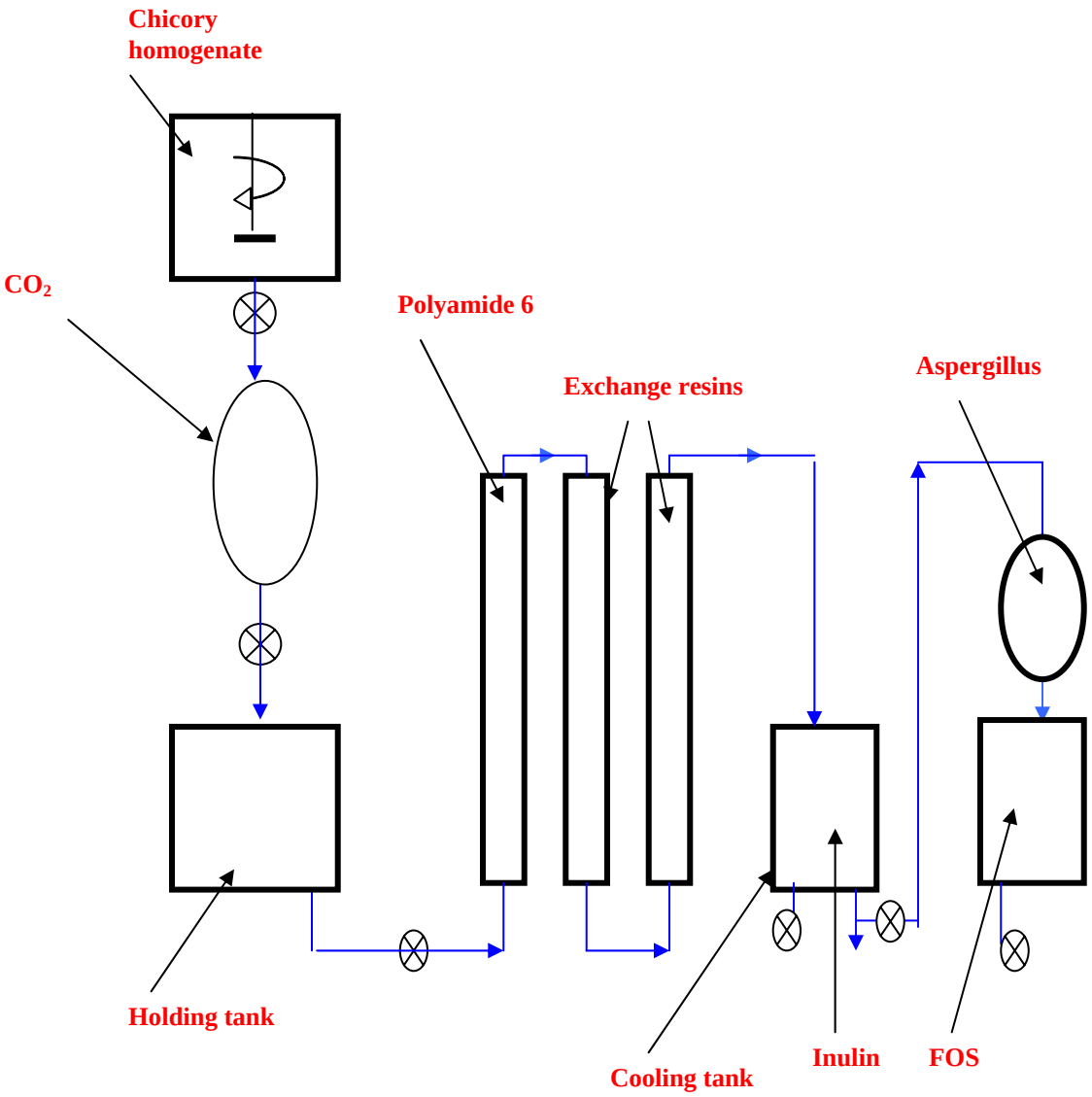

Figure 5.2: Proposed inulin and fructooligosaccharide production using a continuous system.

5.1.3 Materials and Methods

5.1.3.1 Materials

Acetic acid, sodium acetate, sodium chloride, magnesium sulphate heptahydrate, polyethyleneglycol (PEG) 20000, sucrose, K_2CrO_2, $Na_2CO_3.10H_2O$ and sodium thiosulphate were purchased from Merck (South Africa (Pty) Ltd). Bradford reagent, Coomasie Brilliant Blue R staining solution, commercial inulin, dialysis tubing, ferrous sulphate heptahydrate and yeast extract were purchased from Sigma (South Africa (Pty) Ltd). Incubating and shaking procedures were performed using an Orbital incubator shaker supplied by labcon (South Africa). Liquid media were sterilized using a Rexall vertical autoclave (Rexall industries CO. Ltd, Taiwan). Diethylaminoethyl cellulose (DEAE) DE 23 was purchased from Whatman (England). The *Aspergillus niger* inoculum was supplied by the Department of Biochemistry, Microbiology and Biotechnology (Rhodes University. Grahamstown. South Africa).

5.1.3.2 Methods

5.1.3.2.1 *Aspergillus niger* growth

Aspergillus niger, cultivated on potato dextrose agar (PDA) (Merck) at 30°C for 5 days, was obtained from a stock culture at the Department of Biochemistry, Microbiology and Biotechnology, Rhodes University. To determine its ability for high inulinase production, *Aspergillus niger* was grown in a culture incubated in an Orbital incubator shaker at 30 °C and 140 rpm for 4.5 days, using media at pH 4.5 (3.6 % $NH_4H_2PO_4$, 0.21 % KCl, 0.15 % $MgSO_4.7H_2O$, 0.03 % $FeSO_4.7H_2O$, 0.3 % yeast extract) (Ohta *et al.*, 1993) containing 6 % inulin. The liquid media were autoclaved at 121 °C for 20 minutes before inoculation with *A. niger* spores.

The biomass was measured according to the method of Langvad, (1999), where *Aspergillus niger* colony was cut out from the inoculum plate and transferred to a 50 ml

Schott bottle containing 50 ml distilled water. An ultraturex was then used to homogenize the fungus for 30 seconds. Wells A1 to A10 and B2 to B10 of the microtiter plate were filled with 180 µl of the growth medium, followed by 20 µl of sterile distilled water. Wells A2 to A10 and B2 to B10 were then inoculated with 20 µl of the homogenate from the ultraturex. Well A1 was treated as a blank. The plate was read on the Power wave at 630 nm for the duration of 2.5 days at 12 hour intervals until the cells reached confluence.

5.1.3.2.2 Protein determination

The protein was quantified by the Bradford method (Bradford, 1976). Bradford reagent (250 µl) was added into a microtiter plate followed by the growing *A. niger* culture (5 µl). The absorbances were read at 595 nm and the protein concentration determined from the standard curve (*Appendix 5).*

5.1.3.2.3 Inulinase assay

Sampling of the *Aspergillus niger* cultures was performed under sterile conditions. The culture filtrates were assayed for inulinase activities at 12 hour intervals by incubating the centrifuged growing *A. niger* cultures (1 ml) and 2 % (w/v) inulin (4 ml) in 0.1 M acetate buffer, pH 5 (as described by Jing *et al*., 2002). The blank contained boiled crude enzyme and substrate. The amount of released reducing sugar was estimated by the Somogyi-Nelson method (section 2.2.2.1). One inulinase unit is the amount of enzyme that forms 1 µmol fructose per minute.

5.1.3.2.4 Partial purification of inulinase (DEAE DE 23 ion exchange column)

All experiments were carried out at 4 °C. The *Aspergillus niger* culture filtrate was centrifuged (13, 000 × g, 20 min) and the pH adjusted to 5 as described by Nakamura *et al*. (1994). The crude enzyme was concentrated to one-ninth of its original volume in a dialysis bag surrounded by a thick layer of polyethylene glycol (20000). The

concentrated enzyme (2 ml) was loaded onto a DEAE DE 23 column (1.3 × 23 cm) previously equilibrated with 0.02 M acetate buffer pH 6. The DEAE DE 23 resin was prepared as follows: It was regenerated by suspending in HCl (100 ml; 0.5 M) and stirred for 30 minutes. The resin was allowed to settle, the HCl decanted and washed with dH_2O. The resin was then re-suspended in NaOH (100 ml; 0.5 M) and stirred for 30 minutes, after which, it was washed with dH_2O until the pH of the washings approached 7. The ion exchanger was subsequently put in a beaker and equilibrated with 0.02 M acetate buffer pH 6. The ion exchange slurry was then poured into a column and allowed to settle overnight under a flow of 0.02 M acetate buffer pH 6 (0.8 ml/min).

The column was eluted at a flow rate of 0.8 ml/min stepwisely with 0.1 M NaCl (in 0.02 M acetate buffer pH 6) until the absorbance at 280 nm reached a baseline, followed by 0.2 M and 0.3 M NaCl (in 0.02 M acetate buffer pH 6) consecutively. Fractions (4 ml/tube) were collected and monitored for protein and inulinase activity.

5.1.3.2.5 SDS-PAGE

The molecular weights of the enzyme were determined by SDS-PAGE according to the method of Laemmli (1970). The sample (10 µl) from the DEAE ion exchange column, together with SDS-PAGE standard molecular weight (26.6 – 116 KDa) markers (10 µl) were electrophoresed on 12 % SDS-PAGE at 250 V. The gels were stained with Coomasie Brilliant Blue R staining solution, and then destained in methanol-acetic acid-water (1:1:8 v/v/v).

5.1.3.2.6 Silver staining

For enhanced detection silver staining was used, according to the method of Switzer (1979). The gels were fixed in 10 % ethanol and 5 % acetic acid (30 min) and then transferred to an oxidiser solution made up of K_2CrO_2 (0.0032 M; 250 ml) and 55 % nitric acid (57 µl) for 10 minutes. They were washed with water (20 min) until the yellow colour disappeared and treated with silver nitrate (0.2 g in 100 ml) for 10 minutes. The

gels were developed in a solution made up of $Na_2CO_3.10H_2O$ (20g in 250 ml), formaldehyde (375 µl) and sodium thiosulphate (0.1 g).

5.1.3.2.7 Temperature profile of the inulinase

Temperature optimum for inulinase was carried out over a temperature range of 24 to 80 °C. The reaction mixture was prepared as previously described in section 5.1.3.2.3. The reaction was initiated by the addition of inulin and the inulinase activity monitored according to the assay in section 5.1.3.2.3.

5.1.3.2.8 pH profile of the inulinase

The effect of pH was determined over the pH range 3.5 to 6.5 (0.1 M acetate buffer), 6.8 to 7.7 (0.1 M phosphate buffer) and 8 to 8.9 (0.1 M Tris-HCl buffer). Inulinase assays were carried out in reaction mixtures containing 0.5 ml buffer of the desired pH, 0.25 ml 1 % inulin solution (in water) and 0.25 ml enzyme solution.

5.1.4 Results and Discussion

5.1.4.1 Inulinase production from *Aspergillus niger*

Aspergillus niger used in this study demonstrated appreciable growth on inulin, and enzyme production was observed after the onset of the stationary phase, between 60 and 96 hours (Figure 5.3). The delay between the completion of cell growth and appearance of the first sample containing enzyme activity is not uncommon for inulin hydrolysing enzymes. Cruz *et al*., (1998) managed to show that maximum enzyme liberation from *A. niger* 245 occurs between 48 and 60 hours, and noted that in other organisms the maximum enzyme activity is reached after72 hours. These workers offer no explanation for their delayed enzyme secretion. Nevertheless, the biomass and enzyme secretion trend observed in this study is indicative of trends cited by Siebel, (1992) who noted that cell

mass is produced most effectively when the cells are in the growth phase, while enzyme formation rates may be highest when the growth of cells has virtually stalled.

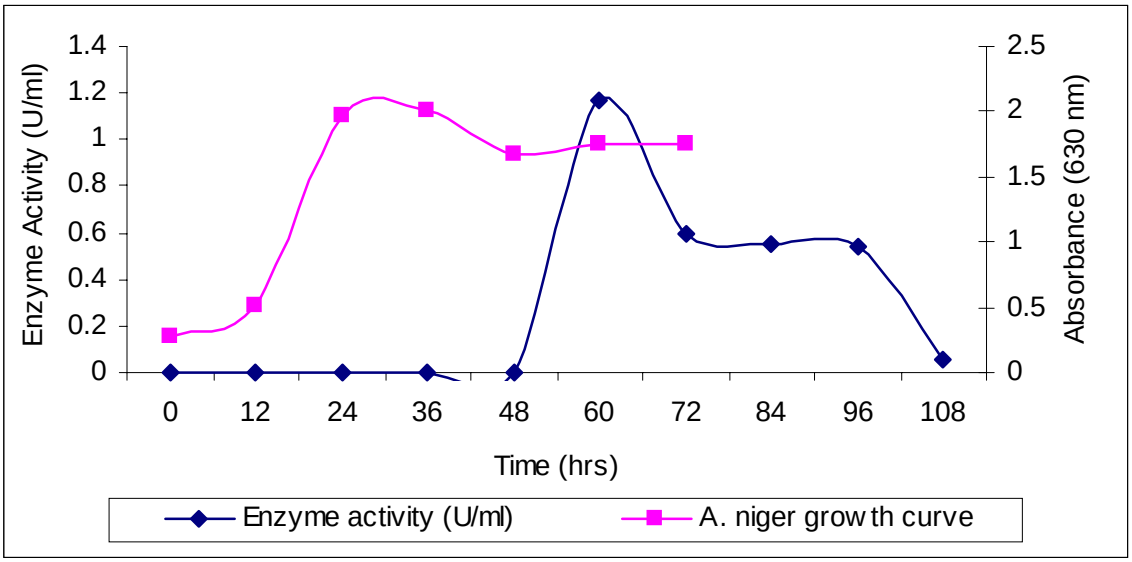

Figure 5.3: Inulinase activity and biomass yield of *Aspergillus niger* grown on inulin. The values are averages of duplicate experiments.

In our opinion the delay was probably due to the fact that inulin was not readily available to the fungus, as it is a complex carbon source, and the observed biomass growth was most likely due to the internal storage compounds contained in the spores. Moreover, substrate utilization by *A. niger* has been shown to be a complex phenomenon that is most probably connected with transporting proteins responsible for carbohydrate transfer from the media into cells (Bizukojc and Ledakowicz, 2004). During the stationary phase, the fungus was in a nutrient deprived state as the internal storage compounds would have been exhausted, as a result the fungus responded by producing enzymes (inulinases) that would simplify the polymer and render it bioavailable, hence the sudden appearance of enzyme activities. The use of inulin as a carbon source was mainly as a result of its frequently proven inulinase induction characteristics (Jing *et al*., 2002).

The *Aspergillus niger* used in this study gave much higher enzyme activities (1.168 U/ml) (figure 5.3) as compared to 0.3 U/ml cited by Vandamme and Derycke, (1983).

5.1.4.2 Partial purification of inulinase (DEAE DE 23 ion exchange column)

The concentrated enzyme extract (1.5 ml) (section 5.1.3.2.4) was loaded into the ion exchange column and eluted at a flow rate of 0.8 ml/min (figure 5.4). After ion exchange chromatography the protein concentration, determined by the Bradford assay, had decreased to 0.21 mg/ml, which made any subsequent purification steps very difficult. Table 5.1 summarizes the purification steps.

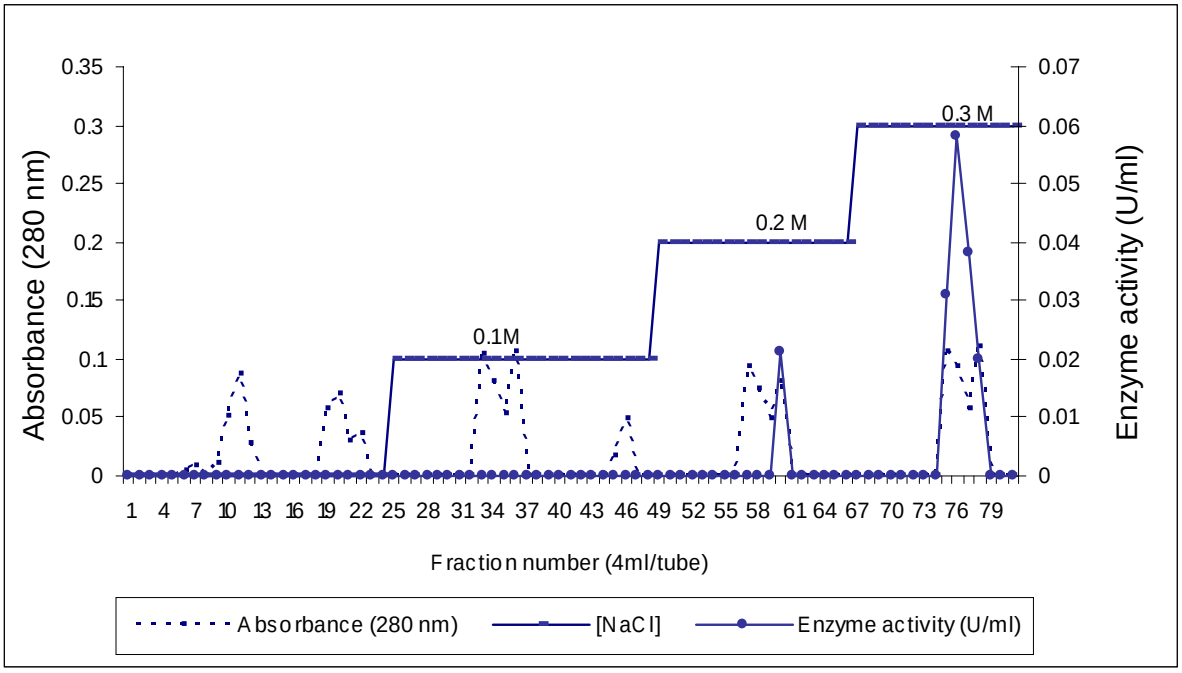

Figure 5.4: DEAE column chromatography (1.3 × 23 cm) profile of inulinase from *Aspergillus niger*.

The decrease in specific activity (table 5.1) was, however, not discouraging as extensive purification of inulinases is not an industrial prerequisite. Both intact and lysed cells, as well as the free culture liquid, relatively display high activity (as observed in this study, table 5.1) (Vandamme and Derycke, 1983). During commercial production of inulinases from culture filtrates, there is partial purification by ion exchange chromatography, concentration and immobilization for the continuous production of fructose syrups (Nakamura *et al.*, 1995).

Table 5.1: Purification table of inulinase-like enzyme from *Aspergillus niger*.

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification factor (fold)
Crude extract	90	105.12	3.87	27.2	100	1
PEG	10	0.5	2.1	0.24	0.48	0.009
DEAE Cellulose Peak 1	4	0.021	0.32	0.07	0.02	0.003
Peak 2	16	0.147	2	0.074	0.14	0.003

During the initial purification of the enzyme ammonium sulphate fractionation was adopted. However, all activity of the enzyme was lost (results not shown) and therefore this step in the purification was left out. Considering table 5.1, it is quickly noticed that virtually all activity of the enzyme was lost using the PEG concentration step. Nevertheless, it was decided to pursue a partial purification using DEAE cellulose.

5.1.4.3 SDS-PAGE and silver staining

Due to the small amount of partially purified enzyme from the ion exchange column, no characteristic bands could be detected using SDS-PAGE and even the more sensitive silver staining. The inulinase nature of the enzyme remains a speculation as the crude enzyme extract and the effluent from the ion exchange column hydrolysed inulin (figure 5.4). Due to these observations, the enzyme was referred to as “inulinase-like” enzyme.

5.1.4.4 Temperature profile of the inulinase

The partially purified inulinase (after DEAE) showed maximum activity at 30 °C (figure 5.5). Published data on inulinase temperature optimum range between 40 and 60 °C (e.g. inulinases from *Talaromyces flavus* range between 40 and 50 °C; *Penicillium sp* secrete inulinases that are active at 45 and 50 °C; *Aspergillus niger sp* inulinases have temperature optima between 45 and 60 °C (Vandamme and Derycke, 1983). The

temperature optimum obtained from this study was similar to that observed for inulinases isolated from *Debaromyces cantarelli*, which was found to be at 30 °C (Vandamme and Derycke, 1983). There is speculation that the *Aspergillus niger* used in this study had become attenuated as it had been subjected to frequent culture transfers for sometime.

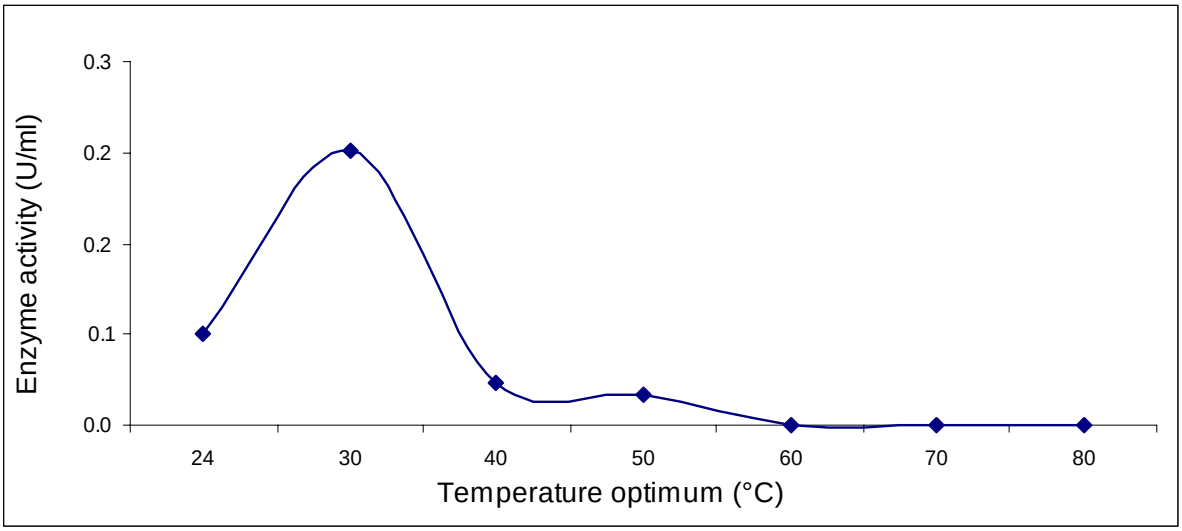

Figure 5.5: The temperature profile for inulinase activity. The values are averages of duplicate experiments.

This observation was noted by Vingaard *et al*., (2003), that certain fungal strains develop abnormal morphology. Altered morphology has a direct bearing on the secondary metabolites of a particular fungus subjected to such conditions or loss of sporulation after several passages through artificial media. In some instances the *Aspergillus niger* inoculum used in this study failed to sporulate suggesting that the fungus had become attenuated.

5.1.4.5 pH profile of the inulinase

The enzyme was active at three different pH values, namely pH 4, 6 and 7.7. pH 7.7 showed the highest enzyme activity at 0.514 U/ml (figure 5.6). The presence of isoenzymes, due to multiple pH optima observed in figure 5.6, could not be ruled out, which points further to the speculation that the *Aspergillus* used in this study may have attenuated over a period of time.

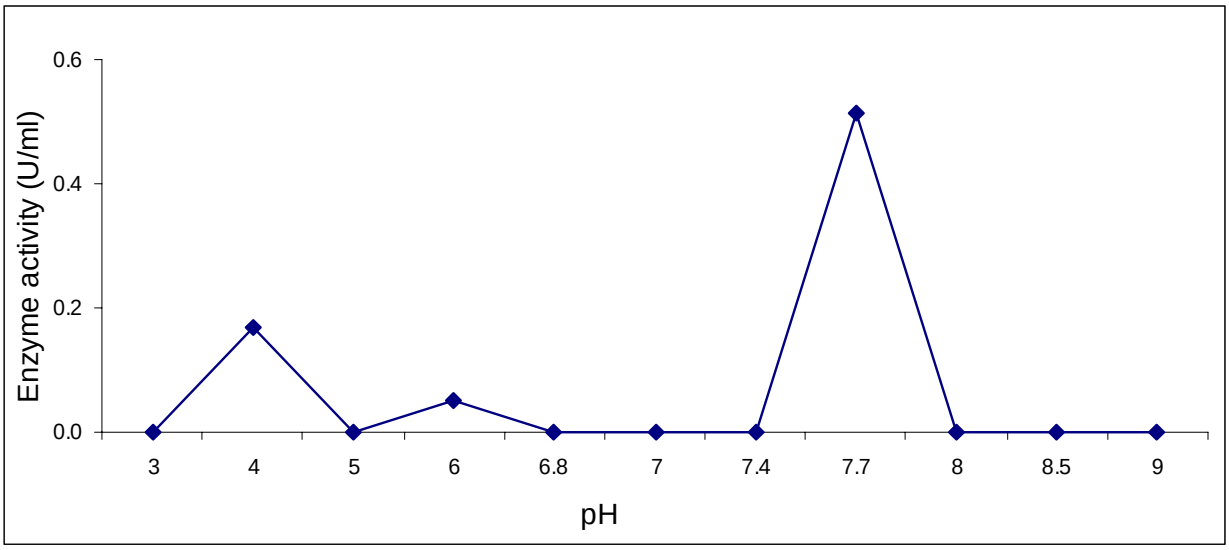

Figure 5.6: pH profile for inulinase. The values are averages of duplicate experiments.

5.1.4.6 Summary

The culture filtrate exhibited an enzyme activity of 1.17 U/ml, which was the highest throughout the study. The crude extract had an optimal temperature and pH at 30 °C and 7.7 respectively (other fraction hydrolyses activities were noticed at two alternative pH values, 4 and 6 suggesting the presence of isozymes). The enzyme proved to be very unstable as it progressively lost its total activity (with diminishing recovery) during ion exchange chromatography. SDS-PAGE of the ion exchange fractions did not reveal any inulinase associated bands and was subsequently followed by silver staining which was not successful either. Thus, the nature of the enzyme being an inulinase remains a speculation, despite its ability to hydrolyze inulin.

Chapter 6

General discussion and conclusion

Since their discovery as functional food ingredients, inulin and oligofructose have continued to attract interest from the food industry and their exploitation has resulted in a number of health products. Inulin and fructooligosaccharides have been discovered to be beneficial food ingredients in as far as the human intestinal integrity is concerned. Their ability to resist degradation in the small intestine is largely due to their β 2→1 glycosidic linkage which renders them unsusceptible to hydrolytic cleavage (as intestinal enzymes target α-configured compounds). Upon reaching the large intestine (unhydrolyzed) they selectively stimulate the growth of beneficial bacteria (bifidobacteria) resident in the colon while preventing pathogenic microbial (clostridia, bacteroides and fusobacteria) growth. Bifidobacterial proliferation is accomplished through inulin fermentation which results in the production of gases (H_2, CO_2 and CH_4), lactic acid and short chain fatty acids (particularly butyrate and propionic acid). The colonic mucosal integrity is consequently maintained by butyrate while propionic acid modulates carbohydrate and lipid metabolism (ultimately regulating serum cholesterol levels) (Roberfroid, 2002; Cherbut, 2002).

As functional food ingredients, inulin and oligofructose have been easily combined with other food materials without compromising their overall flavours and textures. In fact, inulin has been shown to improve mouthfeel, taste and texture of food. Moreover, it has the ability to act as a fat replacer, a feature that can be exploited for the benefit of cholesterol patients (Franck, 2002 b).

As fructose is approximately 2 times sweeter and less cariogenic than normal table sugar (sucrose), fructooligosaccharides with low degree of polymerization (average DP 4) have been used for sweetening purposes. Furthermore, the fact that fructose is less viscous and has higher solubility properties means that it can be metabolized in low levels and

without the need for insulin, a feature that is most beneficial to diabetics (Passador-Gurgel, 1996).

Chicory S.A, one of the largest chicory (primary source of inulin, amongst others) producing companies, focuses mainly on coffee related products and as a result is looking at diversifying its portfolio by participating in markets associated with inulin. The purpose of this study was therefore to develop processes which could be industrially adapted for an economically viable inulin extraction and purification procedure with subsequent fructose production as an alternative sweetener.

The development of inulin and extraction procedures without due compromise to the polymer had to be considered. In as far as inulin extraction was concerned, its physicochemical properties were maximally exploited. As inulin is soluble at high temperatures (precipitating in ambient temperatures), hot water extraction (70 °C) was utilized to isolate most of the inulin in its raw form. However, before inulin extraction could be undertaken, a quantitative assay had to be developed. Hydrochloric acid (0.8 M) was adequate to hydrolyze the inulin into monomeric fructose units which could then be assayed by the standard Somogyi-Nelson procedure. Heating the homogenized chicory root in distilled water (1:5 w/v) at 70 °C for 1 hour, whilst stirring resulted in high recoveries of raw inulin. Heating the homogenate at 80 °C and above resulted in inulin yields that were comparable to those obtained at 70 °C. The optimized extraction process resulted in 80.2 % inulin recovery and 11 % yield.

Different roots are bound to yield varying quantities of inulin due to factors such as time of harvest, shelf storage and temperature. Cold temperatures induce endogenous inulinases which compromise the degree of polymerization of inulin, with subsequent production of fructooligosaccharides and free fructose. Under such conditions the root remobilizes accumulated fructans, through exohydrolases, to produce simple sugars as they act as cryoprotectants (Ritsema and Smeekens, 2003).

Pure inulin is odourless, colourless and tasteless except for fructooligosaccharides which might have slight degree of sweetness (Franck, 2002 b). The raw inulin initially extracted had a deep brown colour, a caramel smell and severe astringency. All these components were most probably as a result of secondary metabolites, especially tannins and sesquiterpene lactones. The presence of pectins were suspected due to gelling properties of the liquid squeezed from an unhomogenized chicory root.

An optimized carbonation process was used to exclude the majority of colour and bitter principles. After adding 0.1 M $Ca(OH)_2$ to the chicory homogenate, CO_2 gas was bubbled through the solution for 10 minutes, and this was enough to remove 90 % of the homogenate's colour and 97 % of the tannins. Bubbling CO_2 for more than 10 minutes did not result in any further colour removal.

Carbonation was followed Polyamide 6 powder (60 g) which managed to remove 63.6 % of the colour and 92 % tannins. Ion exchange (batch wise) purification of the raw chicory extract, using Dowex 50 WX 8 (cation exchanger) and Dowex® 4 (anion exchanger) resulted in 67 % colour removal. Cation exchange resins resulted in negligible colour removal. This observation does not mean that cation exchangers are not necessary, as they still participate in the removal of inorganic salts.

Purification procedures were subsequently linked to effect a continuous tandem purification process so as to assess the viability of a commercial industrial purification system. Three columns were interconnected and operated at 24 °C at a flow rate of 1 ml/min. Prior to passing the chicory homogenate into the columns, carbonation was used to remove the non sugars and colour as mentioned above. Technically, 1 ml/min was an unsatisfactory flow rate as it was time consuming and efforts to increase it were unsuccessful. The procedure was therefore abandoned in favour of a tandem continuous batch wise process. Tandem continuous purification processes resulted in inulin samples that contained less than 1 % tannin and colour.

Mixtures of simple sugars comprised of mono, di and oligosaccharides of DP < 9, are sufficiently resolved by HPLC, however, limitations arise as soon as complex mixtures are analysed. As a result, for complete resolution of all carbohydrates, more than one HPLC column is needed (Doughty, 1995).

The extracted inulin was initially fractionated on Sephadex G-50, to separate it into different DP's. The fractions collected were assayed for free fructose and inulin and a chromatogram generated. Fractions constituting individual peaks were pooled for further analysis.

Analysis of the inulin fractions from Sephadex G-50 was carried out using flourophore assisted carbohydrate electrophoresis of oligosaccharides (FACE). Characterization of fractions using FACE was not successful as no bands could be detected on the polyacrylamide gel even when the pore size was slightly increased. Thin layer chromatography was subsequently used to try and identify the inulin's polymeric distribution. Monosaccharides (fructose) and oligosaccharides that range between DP 2 and 5 were sufficiently resolved, however, spots with highly polymerized oligosaccharides did not migrate. TLC was thus not quantitative in the characterization of inulin.

Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF) was ultimately used. MALDI TOF spectrometry has a few advantages such as tolerance for impure samples and moderate fragmentation on the analyte (Harvey, 1999).

Spectra resulting from the raw sample showed a mass to charge ratio (m/z) that ranged from less than 1000 to 3607.96 translating to a degree of polymerization that varied from 2 to 20. The pure sample on the other hand showed DP values that ranged from 2 to 27. However, native inulin has a maximum DP of 60 (Roberfroid, 2002), which means that the inulin extracted in this study had been compromised. Due to the chicory roots being stored at -10 °C, it is possible that the polymer was degraded. As the chicory roots are normally harvested at 35 weeks (Luckman and Rossouw, 2003), the period when inulin

biosynthesis is still active, it possible that the observed DP, from the MALDI TOF spectra is due to the action of induced exohydraolases (through defoliation and cold temperatures, during storage) which cleave the partial inulin polymer. Results from this study suggest that for the isolation of inulin with maximum DP, chicory roots will have to be processed soon after harvest. Ritsema and Smeekens, 2003, observed that defoliation, dormacy and prolonged cold storage induced exohydrolases that cleave inulin.

The industrial production of high fructose syrups from inulin is generally effected through the use of microbial inulinases (Vandamme and Derycke, 1983). *Aspergillus niger* is the frequently used microorganism as it offers ease and convenience of inulinase recovery (Nakamura *et al*., 1994).

A.niger grown on inulin produced inulin hydrolyzing enzymes with enzyme activities of 1.17 U/ml. Optimum enzyme production was observed after 60 hours. The crude inulinase like enzyme showed maximum temperature and pH activities at 30 °C and 7.7 respectively.

Chromatography of the crude extract on Diethylaminoethyl cellulose (DEAE) DE23, after concentrating with polyethylenglycol (PEG) 20000) revealed two peaks with inulinase activities of 0.005 U/ml and 0.009 U/ml respectively. Fractions with inulinase activities were not fractionated further due to low protein contents and enzyme activities.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining of the DEAE fractions which showed enzyme activity did not reveal any bands. As a result, the inulinase nature of the isolated enzyme remains a speculation, despite the fact that it could hydrolyze inulin.

Vandamme and Derycke, (1983) have noted that extensive purification of inulinases is unnecessary since the cell free culture and both lysed and intact cells show sufficiently high enzyme activities. Based on the observations of these authors and the results obtained from this study, it is recommended that enzymes obtained from optimized

culture filtrates be used for immobilization processes for the production of high fructose syrups.

This project was successful in that, inulin extraction procedures from chicory roots were efficiently optimized. Apart from extracting inulin, FOS were simultaneously isolated, FOS recovery observed in this study is most probably due to the inulin depolymerization as a consequence of exohydrolases induction. It was observed that early harvest and prolonged cold storage result in a cleaved polymer, and thus, logical recommendations towards the recovery of high DP inulin could be made. The study has clearly established that for high inulinase yields with high enzyme activities, in the hydrolysis of inulin, mutant microorganisms may have to be utilized.

Future work involves:

Scaling up of inulin production processes
Over expression of the inulinase
Immobilization studies of the partially purified enzyme extract for continuous fructose syrup production.
Synthesis of high inulin polymers from sucrose using transfructosylating enzymes

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Appendices

Appendix 1: Fructose standard curve

Table 1: Preparation of fructose standard curve (from 1 mg/ml stock solution)

Tube no	Blank	1	2	3	4	5
Fructose standard (ml)	0	0.05	0.1	0.15	0.2	0.25
dH_20 (ml)	6	5.95	5.9	5.85	5.8	5.75
Cu Reagent (ml)	1	1	1	1	1	
Heat at 70 °C for 15 minutes						
Arsenomolybdate (ml)	1	1	1	1	1	1

Leave to stand for 5 minutes. Absorbance read at 510 nm.

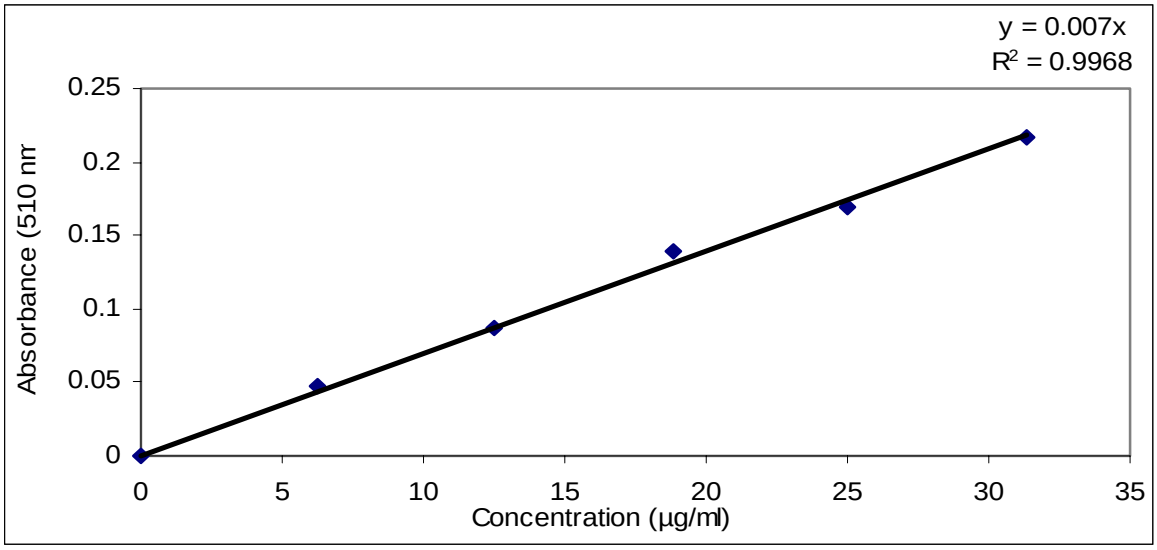

Figure A1: Fructose standard curve.

Appendix 2: Effect of concentrated HCl on the Somogyi-Nelson assay.

Table A2: Effect of concentrated HCl on the fructose standard curve

Tube no	Blank	1	2	3	4	5
Fructose standard (ml)	0	0.05	0.1	0.15	0.2	0.25
dH_20 (ml)	6	5.95	5.9	5.85	5.8	5.75
Cu Reagent (ml)	1	1	1	1	1	
HCl (10 M) ml	0	0.05	0.05	0.05	0.05	0.05
Heat at 70 °C for 15 minutes						
Arsenomolybdate (ml)	1	1	1	1	1	1

Readings obtained

Sample	Absorbance (510 nm)
Blank	0
1	0
2	0
3	0
4	0
5	0

Appendix 3: Tannin standard curve

3.1 Preparation of the Folin Dennis reagent.

Sodium tungstate (25 g), 5 g phosphomolibdic acid and 12.5 ml orthophosphoric acid were added into a 500 ml flask containing 187.5 ml distilled water. The flask was refluxed for 2 hours, allowed to cool and made up to 250 ml and stored in the dark.

Na_2CO_3 solution was prepared in 80 °C dH_20

Table A3: Preparation of tannin standard curve

Tube no	Blank	1	2	3	4	5
Tannic acid (ml)	0	0.06	0.12	0.18	0.24	0.3
dH_20 (ml)	2.8	2.74	2.68	2.62	2.56	2.5
Folin Dennis reagent (ml)*	0.2	0.2	0.2	0.2	0.2	0.2
35 % Na_2CO_3 (ml)	0.4	0.4	0.4	0.4	0.4	0.4
dH_20 (ml)	0.6	0.6	0.6	0.6	0.6	0.6

Incubated at room temperature for 1 hour and the absorbance read at 725 nm

Reaction mixtures were vortexed immediately after adding the Folin Dennis reagent.

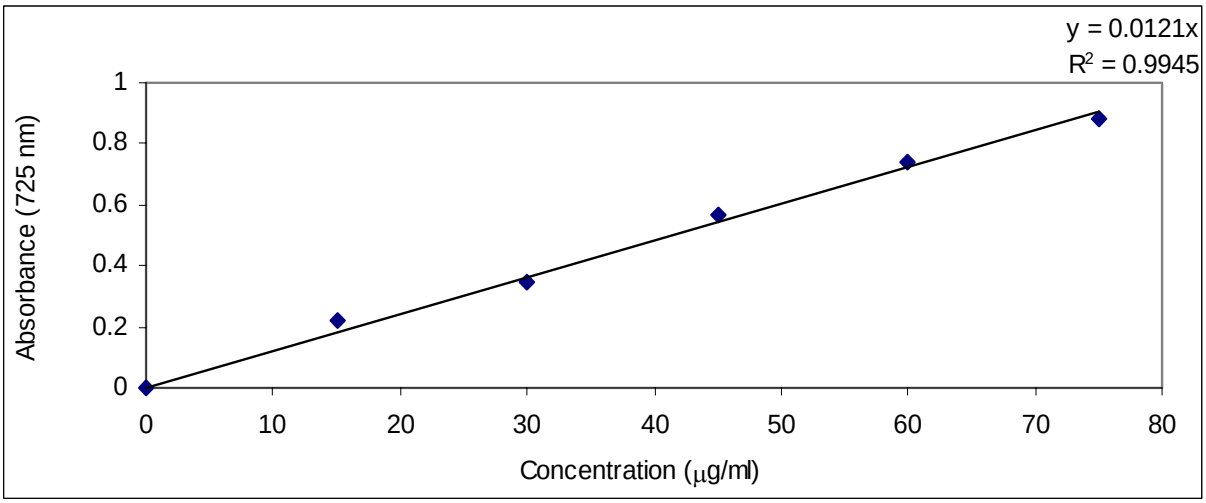

Figure A2: Tannin standard curve

Appendix 4: Degree of polymerization conversion curve.

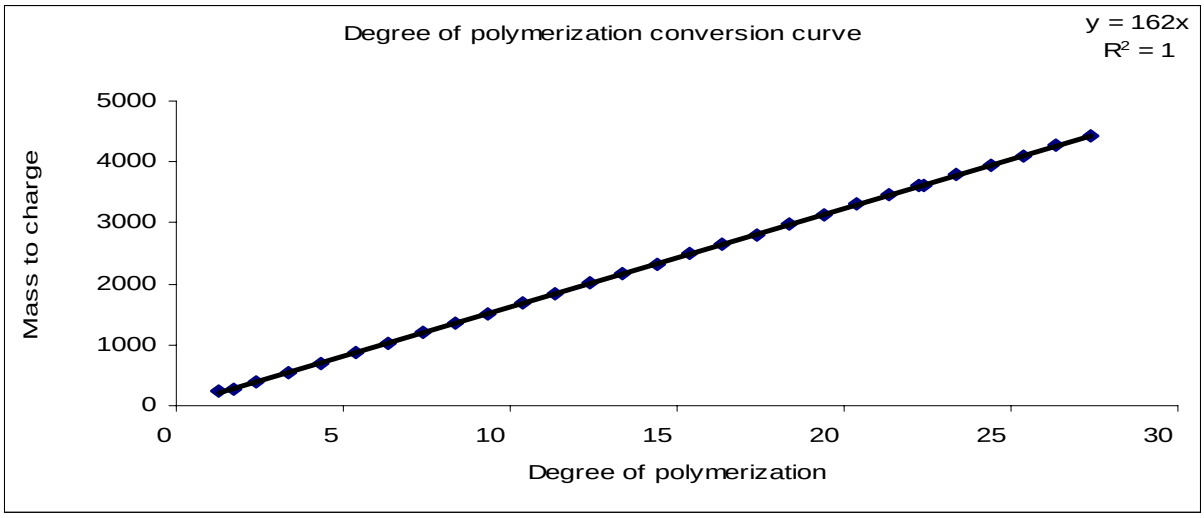

Figure A3: Degree of polymerization conversion curve. The molecular weight distribution was calculated by dividing m/z by 162, except for the first value (divided by 180).

Appendix 5: Bradford Assay

Table A4: Preparation of the protein standard curve

Protein concentration (mg/ml)	BSA stock solution (µl)	dH20 (µl)	Bradford reagent (µl)
0	0	5	250
0.4	1	4	250
0.8	2	3	250
1.2	3	2	250
1.6	4	1	250
2	5	0	250

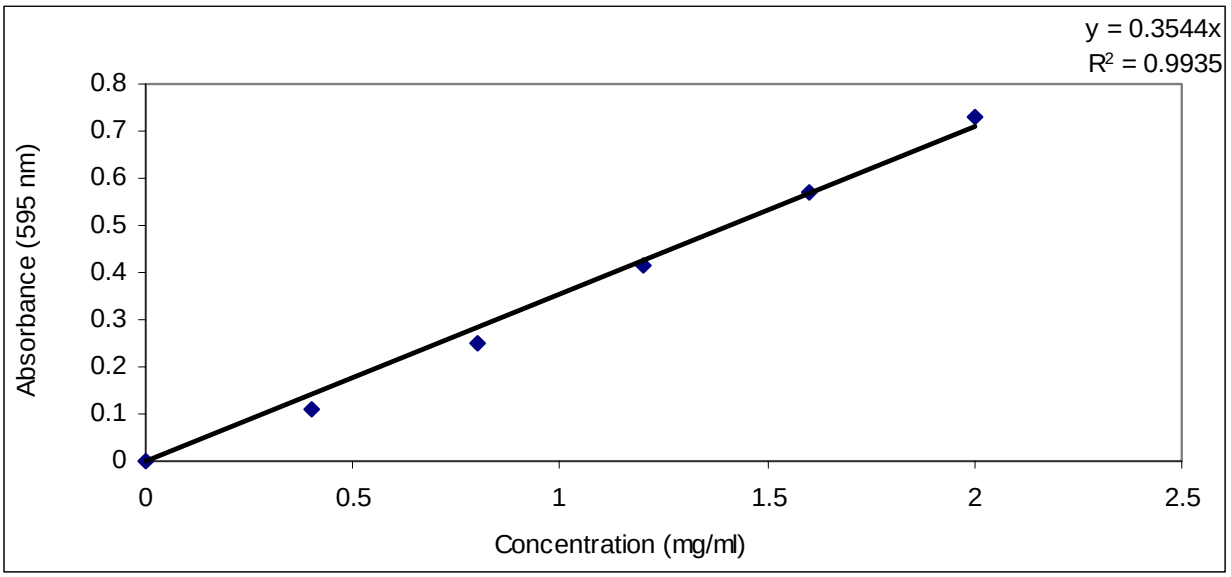

Figure A4: Protein standard curve.